DISSERTATION

SEX-DEPENDENT FUNCTION AND REGULATION OF THE HYPOTHALAMIC PITUITARY ADRENAL AXIS

Submitted by

Ashley L. Heck

Department of Biomedical Sciences

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Doctoral Committee:

Advisor: Robert Handa

Gerrit Bouma
Shane Hentges
Gregory Florant

ABSTRACT

Physiological responses to stressors are largely governed by a neuroendocrine axis, the hypothalamic pituitary adrenal (HPA) axis. Whereas HPA activation is necessary for body wide adaptation to a stressor via the production of glucocorticoids, its excessive or inappropriate activation can increase risk for a number of diseases. Importantly, many of these stress-related diseases exhibit a strong sex bias in prevalence, which may be related to sex differences in the activity of the HPA axis. Thus, the studies described in this dissertation examine sex differences in the regulation and function of the HPA axis in rodents to further unravel the sex-dependent vulnerability often characteristic of stress-related diseases in humans.

In Chapters 2 and 3, sex differences in glucocorticoid negative feedback at the level of corticotropin releasing hormone (*Crh*) in the hypothalamic paraventricular nucleus (PVN), an important factor limiting HPA axis activation, are explored. Results of Chapter 2 indicate that male C57BL/6 mice exhibit a more rapid response of PVN *Crh* expression to the removal of glucocorticoid negative feedback due to androgen actions, likely via upstream regulatory neurons. Results of Chapter 3, alternatively, show more robust glucocorticoid receptor (GR) mediated negative feedback on PVN *Crh* in females, but only on a day of their reproductive cycle when circulating estrogen levels are low. Thus, a complex interplay among androgen/ estrogen actions and glucocorticoid regulatory mechanisms appears to drive sex-dependent PVN *Crh* expression to potentially influence sex-biased HPA activity and stress-related disease risk.

In Chapters 4 and 5, the response of the HPA axis to chronic stress, a factor which is more etiologically relevant for human disease risk, is examined. The results of Chapter 4 demonstrate that female C57BL/6 mice exhibit time-of-day dependent changes in the basal and

acute stress-induced activity of the HPA axis following chronic variable stress (CVS). Male mice, conversely, appear mostly resistant to the effects of CVS on HPA function until socially isolated (Chapter 5). These findings establish an essential foundation for the use of the C57BL/6 mouse, a strain typically more resistant to the effects of CVS, in future studies of sex-dependent HPA axis regulation following chronic stress.

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1.1 Summary

The hypothalamic-pituitary-adrenal (HPA) axis, a neuroendocrine network that controls hormonal responses to internal and external challenges in an organism's environment, exhibits strikingly sex-biased activity. In adult female rodents, acute HPA function following a stressor is markedly greater than it is in males, and this difference has largely been attributed to modulation by the gonadal hormones testosterone and estradiol. These gonadal hormones are produced by the hypothalamic-pituitary-gonadal (HPG) axis and have been shown to determine sex differences in adult HPA function after acute stress via their activational and organizational effects. Although these actions of gonadal hormones are well supported, the possibility that sex chromosomes similarly influence HPA activity is unexplored. Moreover, questions remain regarding sex differences in the activity of the HPA axis following chronic stress and the underlying contributions of gonadal hormones and sex chromosomes. The present review examines what is currently known about sex differences in the HPA axis response to stress, as well as outstanding questions regarding this sex bias. Although it primarily focuses on the rodent literature, a brief discussion of sex differences in the human HPA axis is also included.

1.2 Introduction

The ability of all mammals to cope with any environmental or homeostatic challenge (i.e. stressor), or with perceptual threats to homeostasis, relies upon activation of a neuroendocrine signaling cascade called the hypothalamic pituitary adrenal (HPA) axis. The HPA axis is

[1]Sections 1.1-1.9 of Chapter 1 are modified from: Sex differences in the hypothalamic pituitary adrenal axis' response to stress: an important role for gonadal hormones. Neuropsychopharmacology. Vol. 44, No. 1, pg. 45-58, August 1, 2018.

activated in response to real or perceived stressors and culminates in the production and secretion of glucocorticoids by the adrenal glands. These act upon virtually all tissues to facilitate a body-wide stress response. When acutely elevated by stressors, glucocorticoids induce physiological and behavioral changes that are beneficial and indispensable for survival (Munck *et al*, 1984; Sapolsky *et al*, 2000). However, persistent rises in glucocorticoids due to chronic stress or disease states are detrimental and increase risk for stress-related pathology (De Kloet *et al*, 2005; Holsboer, 2001; Sapolsky *et al*, 2000). Strikingly, women are at twice the risk of men for developing many of these diseases, likely due to sex differences in the function and regulation of the HPA axis (Bangasser and Valentino, 2014). Thus, understanding the nature and causes of such sex differences in the HPA axis following stress has important implications for understanding sex-biased risk for disease.

Much of what is known about sex differences in the stress-induced activity of the HPA axis and their underlying mechanisms comes from studies done in rodents. Such studies have demonstrated that sex differences in the HPA axis can arise from the influence of gonadal hormones during adulthood or during key developmental periods (Green and McCormick, 2016; Handa and Weiser, 2014; Seale *et al*, 2004b, 2005a). Additionally, they support the possibility that sex biases result from sex chromosomal effects, although supporting evidence is currently limited (Arnold, 2009). The present review accordingly focuses on advances revealed from rodent studies in our understanding of sex differences in the stress-induced activity of the HPA axis in adulthood. We primarily outline what is known about how gonadal hormones and sex chromosomes modulate HPA axis activity following acute stress, and then focus on sex-biased HPA axis activity post chronic stress, which is far less well understood. We conclude with a brief

discussion of sex differences in the human HPA axis with the caveat that they are less pronounced than in rodents and are largely dependent on the stress modality.

1.3 Overview of the HPA axis

Activation of the HPA axis

Activation of the HPA axis occurs at the level of the hypothalamic paraventricular nucleus (PVN), which receives varying inputs depending on the nature of the stressor. Some stressors involve an immediate threat to physiological homeostasis and require the rapid relay of peripheral signals to PVN neurons via direct serotonergic or catecholaminergic projections from brainstem nuclei (Handa and Weiser, 2014; Herman and Cullinan, 1997). Other stressors, alternatively, have psychological components that can activate neuroendocrine responses in the absence of a direct threat to survival (Herman *et al*, 2003). These responses involve anticipation of a homeostatic challenge that requires interpretation by higher brain structures in order to assign significance to an external event based on instinctual fears and/or prior experiences with homeostatic challenges (Herman *et al*, 2003). Because these stressors likely involve preparing for a threat to survival rather than immediately coping with the threat itself, there is time for information to first be processed in limbic forebrain regions before it reaches the PVN (Herman *et al*, 2003).

The amygdala is a limbic structure that plays a notable role in activating the neuroendocrine response to various stressors (Jankord and Herman, 2008). It is divided into subnuclei, including the central (CeA), medial (MeA), and basolateral amygdaloid (BLA) nuclei, that all have this excitatory effect (Jankord and Herman, 2008). However, each sub-nucleus is preferentially responsive to specific types of stressors (see (Jankord and Herman, 2008) for review) and may have sex-dependent function (Brunton *et al*, 2011; Mitsushima *et al*, 2006;

Mizukami *et al*, 1983; Toufexis, 2007). Notably, the amygdala sends few direct projections to the PVN (Herman *et al*, 2003; Prewitt and Herman, 1998). Thus, it largely modulates PVN neuronal activity by first innervating limbic or brainstem relay centers (Myers *et al*, 2014). For instance, both the MeA and CeA send GABAergic projections to discrete regions of the bed nucleus of the stria terminalis (BNST) that ultimately function to decrease its GABAergic input to the PVN (Herman *et al*, 2003).

Ultimately, regardless of the nature of the stressor and corresponding neural circuitry, all pathways converge on PVN neurons that initiate the HPA axis response and eventual hormonal response to stress (Herman and Cullinan, 1997) (Figure 1.1).

The HPA axis is activated by a distinct population of hypophysiotropic neurons in the medial parvocellular division of the PVN (Herman *et al*, 2003; Whitnall, 1993). These neurons synthesize a cocktail of neuropeptides, including corticotropin releasing hormone (CRH), vasopressin (AVP), and oxytocin (OT), which are carried through the hypothalamo-hypophyseal portal vasculature where they can stimulate production of adrenocorticotropin (ACTH) by corticotrophs in the anterior pituitary gland (Rivier and Vale, 1983; Schlosser *et al*, 1994; Vale *et al*, 1981). The subsequent increase in the release of ACTH into the systemic circulation can then act in the adrenal cortex to induce the synthesis and release of glucocorticoids (i.e. cortisol in humans, and corticosterone (CORT) in rats and mice). Glucocorticoids act on virtually all tissues to mobilize energy stores, enhance cognition, and increase cardiovascular activity, while suppressing immune, reproductive, and digestive functions (Sapolsky *et al*, 2000). In the short term, stress-related elevations in glucocorticoids permit the beneficial physiological and behavioral changes necessary for acute stress responses. Unfortunately, prolonged elevations in glucocorticoids induced by chronic stress can have detrimental health consequences.

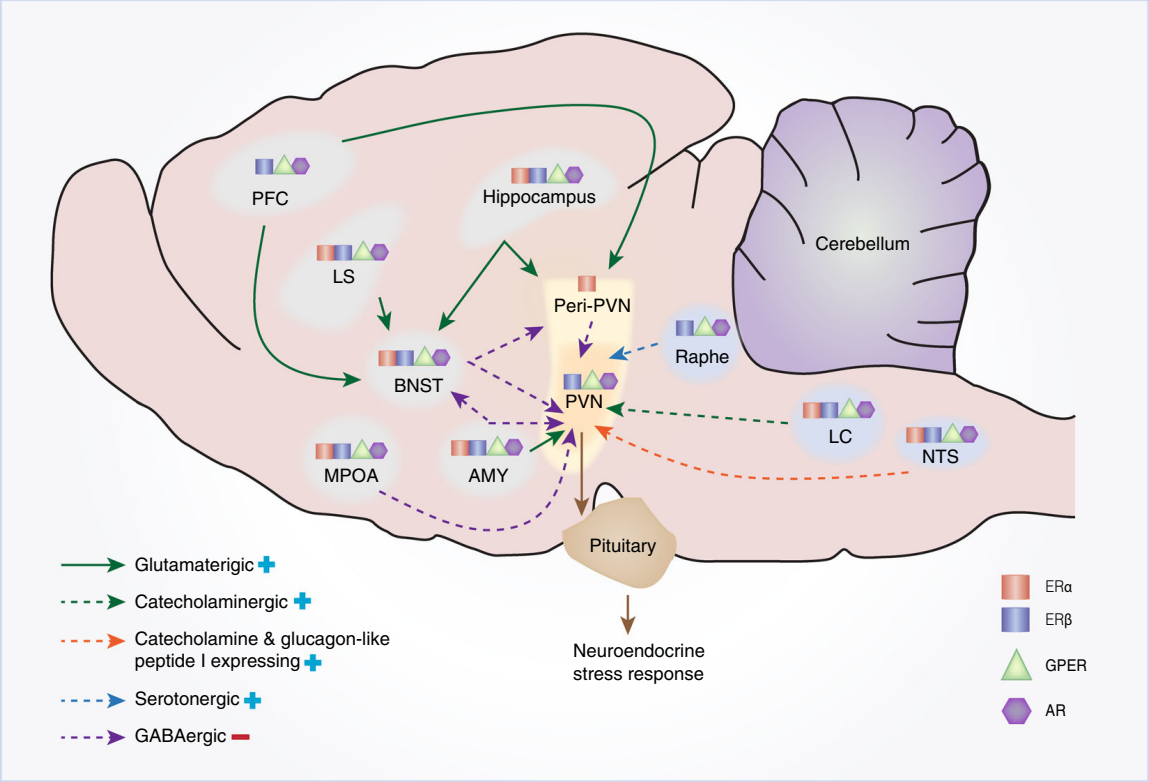

Figure 1.1. Stress-related neuronal inputs to the paraventricular nucleus (PVN) are candidates for gonadal hormone influence on hypothalamic pituitary adrenal (HPA) axis activity. A diagrammatic representation of the major limbic and brainstem structures that send projections to the PVN to either enhance or inhibit the activity of the HPA axis in response to stressors is shown. Projections either directly contact PVN neurosecretory neurons or are indirect in nature and first synapse in the peri-PVN or limbic relay nuclei, such as the BNST. Excitatory and inhibitory projections are indicated by blue plus and red minus signs, respectively. Because their limbic and brainstem origins express androgen and/or estrogen receptors, these projections are potential targets for gonadal hormone modulation of HPA activity. PVN, paraventricular nucleus; PFC, prefrontal cortex; LS, lateral septum; BNST, bed nucleus of the stria terminalis; MPOA, medial preoptic area; AMY, amygdala; LC, locus coeruleus; NTS, nucleus of the solitary tract; Raphe, represents both dorsal and medial raphe nuclei.

Thus, numerous mechanisms exist to enable the tight regulatory control of the HPA axis (De Kloet *et al*, 2005; Holsboer, 2001; Sapolsky *et al*, 2000).

Inhibition of the HPA axis

Glucocorticoid production by the HPA axis is partially maintained within homeostatic limits via neural inhibitory pathways (Figure 1.1). Local brain areas, including the BNST, numerous hypothalamic nuclei, and the region immediately surrounding the PVN (i,e, peri-PVN), project to and directly inhibit PVN neuronal activity (Handa and Weiser, 2014; Herman and Cullinan, 1997). These relay centers contain dense populations of GABAergic neurons. Thus, glutamatergic projections from limbic structures, such as the prefrontal cortex and hippocampus, can inhibit PVN neuronal activation by increasing tone of the inhibitory relay center (Herman *et al*, 2013; Diorio *et al*, 1993). Notably, the involvement of limbic structures in constraining the activity of the HPA axis is largely restricted to stressors with a psychological component (Herman and Cullinan, 1997). The neural inhibition of HPA activity induced by physical stress is far less well understood, but may involve inhibition of excitatory glucagon-like peptide 1 expressing neurons (Herman *et al*, 2012; Zhang *et al*, 2009). Nevertheless, inhibitory neural pathways cannot act alone to inhibit the HPA axis and synthesis of glucocorticoids.

The actions of such inhibitory neural pathways are complemented by a glucocorticoid-mediated negative feedback system (Herman and Cullinan, 1997). In this system, glucocorticoids alter the gain of key HPA regulatory regions, including limbic structures (i.e. hippocampus and prefrontal cortex), the PVN, and the anterior pituitary, to ultimately reduce adrenal glucocorticoid production (Herman *et al*, 2012). Glucocorticoids act through either the mineralocorticoid receptor (MR) or the glucocorticoid receptor (GR) to exert these effects (Reul and de Kloet, 1985). However, the differential expression and affinity for CORT of the MR and

GR give them functionally distinct feedback roles. The MR is mostly found in limbic structures and has a high affinity for CORT (Reul *et al,* 1987; Ahima *et al*, 1991). Consequently, MRs are thought to be occupied when CORT is at basal levels and are important for maintaining low basal glucocorticoid secretion (Reul *et al*, 1987; Reul and de Kloet, 1985; Spencer *et al*, 1998). GR, alternatively, is expressed in limbic areas, the PVN, and the anterior pituitary, and it has relatively lower affinity for CORT (Reul *et al*, 1987; Reul and de Kloet, 1985). It is selectively occupied by stress-induced elevations in CORT levels and, therefore, plays a significant role in reducing HPA axis activity following a stressor (Reul *et al*, 1987; Spencer *et al*, 1998). Accordingly, GR mediates the majority of glucocorticoid negative feedback processes (de Kloet *et al*, 1998). In support of this, GRs regulate two well-established phases of glucocorticoid feedback on the HPA axis: fast (seconds to minutes) and delayed (minutes to hours). Fast feedback involves membrane bound GRs that induce endocannabinoid suppression of the HPA axis (Di *et al*, 2003; Evanson *et al*, 2010), whereas delayed feedback involves a classical nuclear receptor-mediated mechanism (Keller-Wood and Dallman, 1984; Levin *et al*, 1988). In this classical mechanism, unoccupied GR is found in the cell cytoplasm and translocates to the cell nucleus upon ligand binding where it can directly influence gene transcription by binding glucocorticoid response elements and/or by interacting with other transcriptional regulators (Oakley and Cidlowski, 2013). It ultimately adjusts HPA axis activity by altering expression of HPA related genes, such as *Crh* and *Avp* (Aguilera and Liu, 2012; Ferrini *et al*, 1997). A thorough discussion of the mechanisms of GR action can be found in Keller-Wood 2015 (Keller-Wood, 2015). For more detail on steroid hormone receptor function and GR-mediated negative feedback on PVN *Crh* please see addenda sections 1.10 and 1.11, respectively. Please also see addendum 1.12 for a brief section on the circadian and ultradian regulation of the HPA axis.

1.4 Sex differences in HPA axis activity

In rodents, prominent sex differences exist in the HPA axis response to stress. Females typically have a more robust neuroendocrine response to acute stress, as evidenced by their increased CORT and ACTH levels compared to males following exposure to a number of stressors with different modalities (Babb *et al*, 2013; Handa *et al*, 1994a; Iwasaki-Sekino *et al*, 2009; Viau *et al*, 2005). Sex differences at each level of the HPA axis, as well as in regulatory limbic structures, may underlie this sex-biased HPA output. In the PVN, neuronal activation following numerous stressors, as measured by the expression of immediate early genes such as c-Fos, is greater in females (Larkin *et al*, 2010; Seale *et al*, 2004b; Viau *et al*, 2005). Additionally, expression of HPA-related genes is sexually dimorphic. Female rats have been shown to have greater expression of AVP and CRH mRNA in the PVN and greater expression of the ACTH precursor, proopiomelanocortin (POMC) mRNA, in the anterior pituitary following acute stressors than males (Babb *et al*, 2013; Iwasaki-Sekino *et al*, 2009; Seale *et al*, 2004a; Viau *et al*, 2005).

In addition to their greater hormonal response to stress, female rats have been shown to have a delayed return to baseline ACTH and CORT levels after acute stress, indicating sex differences in the negative feedback regulation of the HPA axis (Babb *et al*, 2013; Handa *et al*, 1994a; Iwasaki-Sekino *et al*, 2009; Viau *et al*, 2005). Sex differences are present in neural pathways that may explain this sex-biased inhibition. In limbic structures known to activate inhibitory inputs to the HPA axis, including the frontal cortex, cingulate cortex, piriform cortex, and hippocampus, neuronal activation following acute restraint stress is reduced in females compared with males (Figueiredo *et al*, 2002).

Sex differences also exist in glucocorticoid feedback mechanisms that may similarly contribute to the less robust negative feedback on the HPA axis in females. Glucocorticoid binding is lower in the hypothalamus of female versus male rats, suggesting that females have fewer hypothalamic corticosteroid receptors (Turner and Weaver, 1985). In the pituitary, female rats also have a reduced density of MRs and GRs compared to males (Turner, 1990). Furthermore, regulation of corticosteroid receptors in response to acute stressors varies between males and females in structures regulating HPA activity, including the hippocampus, hypothalamus and pituitary (Karandrea *et al*, 2000, 2002). For example, acute stressors upregulate GR and MR mRNA in the hypothalamus of male, but not female rats (Karandrea *et al*, 2002).

GR knockout studies have further highlighted sex differences in the feedback regulation of the HPA axis by glucocorticoids. In mice with GR selectively depleted in forebrain regions, including the hippocampus, medial prefrontal cortex, and basolateral amygdala, sex differences have been identified in the necessity of GR in these regions for HPA axis regulation (Solomon *et al*, 2012). The loss of forebrain GR results in HPA axis dysregulation in males but not females, suggesting that forebrain GR is largely inconsequential for normal HPA regulation in females (Solomon *et al*, 2012). The same group then examined the effects of selectively deleting GR in the PVN in male versus female mice (Solomon *et al*, 2015). Interestingly, males showed increases in ACTH and CORT responses to acute stress above those of wild type animals, but this response was absent in females (Solomon *et al*, 2015). Accordingly, PVN GR may be necessary for feedback inhibition of stress induced ACTH and CORT in males, whereas GR in other, unexamined areas may be more important in females.

The availability of corticosteroids in brain regions where they can modulate HPA axis function may also influence their sex-dependent actions. Following their release from the adrenal gland, most corticosteroids are bound by corticosteroid binding globulin (CBG), a glycoprotein produced by the liver (De Kloet *et al*, 2005). The role of CBG is to protect corticosteroids from degradation as they are transported in the plasma to their target tissues (De Kloet *et al*, 2005). Upon reaching the target, CBG releases corticosteroids by a number of mechanisms (see (Panagiotakopoulos and Neigh, 2014) for review), as corticosteroids can only bind their intracellular receptors if they are not bound by CBG (Henley and Lightman, 2011). Thus, CBGs modulate the amount of plasma corticosteroids available to act on their targets, and it is important to make the distinction between total plasma CORT levels and bioavailable "free" CORT when considering sex differences in the activity of the HPA axis (Panagiotakopoulos and Neigh, 2014). One study examining sex differences in basal CBG and free CORT levels found that females have approximately twice the binding activity of males, but only slightly higher (not statistically significant) levels of free CORT (Gala and Westphal, 1965). Consequently, the higher basal levels of total CORT in females may be partly buffered by their higher levels of CBG (Gala and Westphal, 1965; McCormick *et al*, 2002). It is also possible that the increased CBG levels contribute to the enhanced basal and stress induce activity of the HPA axis observed in females, because CBG makes CORT less available for the negative feedback mechanisms that shut down the HPA axis. In males, CBG is negatively regulated by acute stress, which makes CORT more available for negative feedback (Tannenbaum *et al*, 1997). Whether or not this is true for females as well remains to be determined. Thus, higher female CBG levels may make them less vulnerable to elevated HPA axis activity following acute stress but further research is necessary.

1.5 Activational effects of gonadal hormones

Adult sex differences in the neuroendocrine response to acute stress are due in part to interactions between the HPA axis and a parallel neuroendocrine network that controls reproduction, the hypothalamic pituitary gonadal (HPG) axis (Viau, 2002). The HPG axis culminates in the production of testosterone and synthesis of estrogens from aromatizable androgens in the testis and ovary, respectively. For more detail on the control of gonadal hormone production see addendum section 1.13. Both testosterone and estradiol can in turn modulate the HPA axis in adulthood (i.e. have activational effects) and contribute to its sex-dependent function, as will be explored in this section. Notably, gonadal steroids also play a role in altering HPA axis function in aging rodents, although this topic fall beyond the scope of this review (Bale and Epperson, 2015).

Gonadal hormone receptors and their localization

Estrogens and androgens influence the activity of the HPA axis in adulthood by acting through estrogen receptors (ERs) and androgen receptors (ARs), respectively (Handa and Weiser, 2014). The androgens, testosterone and its metabolite, dihydrotestosterone (DHT), both bind the AR, whereas the potent endogenous estrogen, estradiol, can activate either of two major ER subtypes: estrogen receptor alpha (ERα) or beta (ERβ). AR, ERα and ERβ all belong to the same family of receptors, nuclear receptor subfamily 3, which also includes GR, MR and the progesterone receptor (PR) (Mangelsdorf *et al*, 1995). All receptors in this subfamily function classically as ligand activated transcription factors (Pawlak *et al*, 2012). They reside as multi-protein complexes in the nucleus or cytoplasm until ligand binding triggers their shuttling to chromatin DNA where they can influence transcription (Pawlak *et al*, 2012). To alter transcription these receptor (1) bind directly to hormone response elements on DNA and/or (2)

interact with other transcriptional regulators (Bennett *et al*, 2010; Paech *et al*, 1997; Pawlak *et al*, 2012). As is the case for other members of nuclear receptor subfamily 3, ERs and ARs can also be found as membrane receptors that have faster (non-classical) influences on neuronal function and/or transcriptional activity by modulating second messenger pathways and ion channels (Foradori *et al*, 2008; Vasudevan and Pfaff, 2008). Moreover, a membrane estrogen receptor, G-protein coupled estrogen receptor (GPER), has been identified that has high affinity for estradiol and can also mediate its rapid effects (Brailoiu *et al*, 2007). Thus, through classical and non-classical roles, gonadal hormone receptors enable gonadal hormones to influence HPA axis activity in a rapid and delayed manner.

The widespread expression of gonadal steroid hormone receptors in key parts of the neural circuitry controlling the HPA axis enables gonadal steroids to modify the neuroendocrine response to stress in concert with changes in reproductive function (Figure 1.1). AR, ERα and ERβ expression accordingly has been found in, cortex, hippocampus, medial amygdala, BNST, and numerous hypothalamic areas, including the medial preoptic area (MPOA) (Simerly *et al*, 1990). GPER is also expressed in the hippocampus and hypothalamic areas (Brailoiu *et al*, 2007). In neuroendocrine PVN neurons that direct the activity of the HPA axis, however, ERβs and GPERs are robustly expressed whereas ARs are limited, suggesting that gonadal steroids modulate HPA activity by distinct mechanisms of action (Bingham *et al*, 2006; Handa *et al*, 1994a). Estradiol can directly alter HPA function by acting in neuroendocrine PVN neurons or indirectly by acting though upstream brain regions that project to the PVN; androgens, alternatively, have been shown to have predominantly indirect effects (Handa *et al*, 1994a). The MPOA and BNST, for example, are particularly important mediators of androgens' actions,

as they have abundant AR expression and project to the PVN and peri-PVN (Handa and Weiser, 2014; Williamson *et al*, 2010; Williamson and Viau, 2007).

The estrous cycle and the HPA axis

Sex differences in the activity of the HPA axis may largely be influenced by HPG-mediated fluctuations in the levels of estradiol that occur across a 4-day estrous cycle in female rodents. Early investigations revealed that, as estradiol concentrations increase throughout the estrous cycle in female rats, so do the basal and stress induced activity of the HPA axis. Accordingly, female rodents in diestrus (low estradiol) are similar to males in that they exhibit low resting glucocorticoid secretion and a relatively quick on-off response to stressors (Herman *et al*, 2016; Viau and Meaney, 1991). Females in proestrus (high estradiol, high progesterone) and estrus (recent exposure to peak estradiol), alternatively, have elevated basal and stress-induced ACTH and CORT (Carey *et al*, 1995; Herman *et al*, 2016; Viau and Meaney, 1991). Notably, elevations in HPA output are greatest on proestrous morning, when estradiol levels are peaking, but elevations in progesterone have not yet occurred. Progesterone appears to reduce estradiol's effects on HPA output. Accordingly, in ovariectomized (OVX'd) female rats replaced with physiological levels of estradiol with or without progesterone to mimic the estrous cycle, estradiol treatment enhances HPA activity more than estradiol and progesterone treatments combined (Viau and Meaney, 1991). Thus, the extent of estradiol's ability to increase HPA output on proestrus depends on whether there is a high or low background level of progesterone (Patchev *et al*, 1994b; Viau and Meaney, 1991). This is well aligned with the findings of rodent studies demonstrating that progesterone treatment alone suppresses HPA axis stress reactivity (Owens *et al*, 1992; Patchev *et al*, 1996).

Female rats in proestrus and estrus also have a delayed return to baseline glucocorticoid secretion following stress (Herman *et al*, 2016; Viau and Meaney, 1991). This may be due to less robust mechanisms of glucocorticoid negative feedback on the HPA axis and/or lesser input from limbic structures that are known to inhibit the HPA axis (Figueiredo *et al*, 2002; Herman *et al*, 2016). Indeed, neuronal activation in cortical and hippocampal areas is lower in proestrous and estrous females than diestrous females (Figueiredo *et al*, 2002). Overall, studies of HPA axis function across the reproductive cycle have highlighted an important role for estradiol in controlling the neuroendocrine response to stress.

Estradiol and the HPA axis

In rodents, estradiol often enhances the activity of the HPA axis. Numerous studies correspondingly report that the removal of most endogenous estrogens by ovariectomy (OVX) decreases stress induced ACTH and CORT levels, while estradiol treatment has the opposite effects (Figueiredo *et al*, 2006; Seale *et al*, 2004b; Viau and Meaney, 1991). However, there are exceptions to this pattern, as some studies demonstrate that estradiol treatment can inhibit the HPA axis (Ochedalski *et al*, 2007; Young *et al*, 2001). Such exceptions will be discussed shortly. Nonetheless, support for the excitatory effects of estradiol has been found at all levels of the HPA axis. In the PVN, estradiol has been shown to increase stress-induced neuronal activation (Larkin *et al*, 2010), as well as *Crh* and *Avp* gene expression (Lunga and Herbert, 2004; Seale *et al*, 2004b). Additionally, estradiol increases POMC mRNA (Seale *et al*, 2004b) and ACTH content (Kitay, 1963) in the pituitary; and it increases sensitivity to ACTH in the adrenal gland (Figueiredo *et al*, 2006). Lastly, estradiol disrupts GR mediated negative feedback on the HPA axis, as it reduces the repression of PVN *Crh* by glucocorticoids (Patchev and Almeida, 1996) and interferes with GR expression and binding in the pituitary and hippocampus (Burgess and

Handa, 1993). Estradiol also upregulates CBG levels (Gala and Westphal, 1966), but this is not necessarily responsible for estradiol's effects on the acute neuroendocrine stress response, since estradiol upregulates the HPA axis even in the absence of CBG changes (Lund *et al*, 2004a).

Other studies show that estradiol inhibits HPA responses to stressors or has no effect (Babb *et al*, 2013; Ochedalski *et al*, 2007; Young *et al*, 2001). Accordingly, under certain physiological conditions, estradiol has been shown to decrease PVN neuronal activation (Figueiredo *et al*, 2006) and *Crh* gene expression (Ochedalski *et al*, 2007; Paulmyer-Lacroix *et al*, 1996). Reports of such apparently opposing effects of estradiol on HPA activity may be due to varying experimental conditions, such as the dose or duration of estradiol treatment. Interestingly, a recent study measuring the effects of estradiol on stress related, anxiety-like behaviors showed that the response to estradiol was opposite if females were OVX'd and fed a standard chow versus a phytoestrogen free diet (Russell *et al*, 2017). HPA axis activity was not measured in this study, unfortunately, but its findings suggest that food composition can greatly affect the way that animals respond to estradiol.

Additionally, it is likely that estradiol will have contrasting actions based on whether ERα or ERβ mediated signaling is invoked (Handa and Weiser, 2014). Whereas selective activation of ERα with propylpyrazoletriol (PPT) increases glucocorticoid secretion following stressors, selective activation of ERβ with diarylpropionitrile (DPN) decreases it in OVX'd female rats (Handa and Weiser, 2014; Lund *et al*, 2005, 2006; Weiser and Handa, 2009). ERα and ERβ also modulate HPA activity via distinct mechanisms of action. ERβ can directly modify PVN action and downstream HPA output, as it is co-expressed by PVN neurons that express CRH, AVP, and OT (Hrabovszky *et al*, 2004; Laflamme *et al*, 1998; Lund *et al*, 2006). Notably, ERβ co-expression with these neuropeptides varies based on the species and neuropeptide

examined. For example, ERβ is found in 2/3 of AVP neurons in the rat PVN, whereas it is expressed in less than 20% of these neurons in the mouse PVN (Hrabovszky *et al*, 2004; Oyola *et al*, 2017). 60-80% of OT neurons, alternatively, co-express ERβ in both rats and mice (Hrabovszky *et al*, 2004; Oyola *et al*, 2017). In contrast to the presumably direct actions of ERβ, ERα may influence HPA activity more indirectly. Although some ERα expression has recently been reported in mouse PVN neurons, very limited expression has been found in the rat PVN (Hrabovszky *et al*, 2004; Oyola *et al*, 2017). More substantial expression of ERα has been found in brain regions that project to the PVN, such as the peri-PVN, BNST, and hippocampus (Laflamme *et al*, 1998; Weiser and Handa, 2009), supporting its indirect effects on HPA activity. Accordingly, studies indicate that ERα can inhibit glucocorticoid negative feedback on the HPA axis by disinhibiting the inhibitory signal from the peri-PVN to the PVN (Weiser and Handa, 2009).

Moreover, estradiol may decrease excitatory inputs to the PVN by binding GPERs. The PVN receives serotonergic projections from the median and dorsal raphe nuclei of the brainstem that generally induce activation of the HPA axis (Figure 1.1) (Van De Kar and Blair, 1999); and estradiol has been shown to partially decrease this effect by desensitizing serotonin receptor signaling in the PVN (Raap *et al*, 2000). Studies in OVX'd rats have correspondingly shown that treatment with a serotonin receptor agonist increases plasma ACTH levels and that estradiol administration limits this effect (McAllister *et al*, 2012). Interestingly, GPER, but not ERβ, has been shown to be necessary for the desensitizing effects of estradiol on serotonin signaling in the PVN (McAllister *et al*, 2012; Rossi *et al*, 2010).

Androgens and the HPA axis

Androgens generally inhibit the activity of the HPA axis. Thus, removal of most endogenous androgens by gonadectomy (GDX) increases stress induced ACTH and CORT secretion, whereas testosterone treatment has the opposite effects (Seale *et al,* 2004a; Seale *et al*, 2004b; Viau and Meaney, 2004). Notably, the reduction of testosterone to the more potent androgen, DHT, is necessary for its suppression of glucocorticoid secretion after stress (Handa *et al*, 2013). Accordingly, inhibition of the enzyme that converts testosterone to DHT, 5α-reductase, with central infusion of finasteride increases stress-enhanced glucocorticoid secretion in male rats (Handa *et al*, 2013). This effect is reversed by treatment with DHT, but not testosterone, indicating that testosterone's inhibition of HPA activity depends on 5α-reductase activity within the central nervous system in males (Handa *et al*, 2013). Moreover, estradiol does not potentiate the effects of DHT on the HPA axis in adulthood as may occur during development (Bingham *et al*, 2011a) and reproductive behaviors (D'Occhio and Brooks, 1980). DHT placed near the PVN in adult, gonadectomized (GDX'd) male rats reduces, whereas estradiol and ERα- selective agonists increase the ACTH and CORT responses to acute stress (Lund *et al*, 2006).

As illustrated for estrogens, the overall effect of androgens on the HPA axis is partially mediated by androgen actions within the hypothalamus. In the PVN, androgens decrease neuronal activation, as well as elevations in CRH and AVP mRNA brought on by varying types of stressors (Lund *et al*, 2004b; Seale *et al*, 2004b; Viau *et al*, 2003). These effects of androgens on PVN function and neuropeptide expression are likely mediated by ARs in limbic structures upstream of the PVN, because neuroendocrine PVN neurons do not express ARs (Bingham *et al*, 2006; Handa *et al*, 2009). The MPOA and BNST, alternatively, are rich in AR expression and

send AR immunoreactivity expressing projections to the PVN (Williamson *et al*, 2010; Williamson and Viau, 2007). Thus, they are favorably positioned to facilitate regulation of the HPA axis by androgens. Accordingly, androgen treatment administered directly to the MPOA decreases neuronal activation and *Avp* gene expression in the PVN, as well as glucocorticoid secretion following acute stress (Viau and Meaney, 1996; Williamson *et al*, 2010). Stereotaxic placement of DHT in the BNST, on the other hand, increases PVN AVP mRNA and stress-induced neuronal activation (Bingham *et al*, 2011b). While further examination is necessary to determine if this unexpected role of androgens in the BNST depends on the type or duration of stress exposure, it supports the idea that androgens can act on brain regions upstream of the PVN to modulate the function of the HPA axis (Bingham *et al*, 2011b).

Androgens also act downstream of the PVN to influence HPA axis activity. At the level of the pituitary, POMC mRNA is downregulated by androgens (Seale *et al*, 2004b). Androgens also decrease pituitary ACTH content (Kitay, 1963) and adrenal CORT content (Malendowicz and Młynarczyk, 1982). Moreover, androgens enhance GR-mediated negative feedback on the HPA axis. This is indirectly supported by evidence that testosterone increases GR binding in the MPOA, an area that mediates some of the inhibitory effects of glucocorticoids on the HPA axis (Viau and Meaney, 1996). It is also supported by evidence that testosterone decreases plasma CBG levels, which makes CORT more accessible to its receptors that facilitate glucocorticoid negative feedback (Viau and Meaney, 2004).

Importantly, most studies examining androgen effects on the HPA axis employ DHT, which has high affinity for ARs, but cannot be aromatized to estradiol (Handa *et al*, 2009). However, DHT, can be metabolized to 5α-androstane-3β, 17β-diol (3β-diol), which has been shown to bind and activate ERβ (Handa *et al*, 2009). Like DHT and ERβ agonists, 3β-diol

inhibits the HPA axis response to stress (Handa *et al*, 2009; Lund *et al*, 2006). Accordingly, administration of either DHT or 3β-diol just above the PVN decreases ACTH and CORT responses to an acute stressor (Lund *et al*, 2006). It has been hypothesized that 3β-diol and DHT, via its metabolism to 3β-diol, act through ERβs in PVN neurons to exert these effects, as rat PVN neuroendocrine neurons do not express ARs (Handa *et al*, 2009). In support of this hypothesis, an ER but not AR antagonist blocks the local actions of DHT and 3β-diol in the PVN (Lund *et al*, 2006). Additionally, 3β-diol has been shown to directly regulate the *Ot* and *Avp* promoters through binding to ERβ (Hiroi *et al*, 2013; Pak *et al*, 2009). Together, these findings suggest that the inhibitory effects of DHT on HPA axis activity may be partially mediated by 3β-diol signaling through ERβ. They also emphasize the importance of further considering the roles ERβ may play in facilitating androgen regulation of the HPA axis.

1.6 Organizational effects of gonadal hormones

Perinatal gonadal hormones and the HPA axis

Sex differences in HPA function appear to also be organized by exposure to gonadal steroids during key developmental periods that program lasting differences in the HPA axis (i.e. organizational effects) (Green and McCormick, 2016) (Figure 1.2). During perinatal development, male rats and mice encounter two surges in testosterone release that can presumably masculinize/ defeminize the brain prior to puberty. One occurs late in gestation (G18 in the rat) and the other occurs shortly after birth (Corbier *et al*, 1992; Weisz and Ward, 1980). Accordingly, if neonatal testosterone is removed by GDX, or if ARs are antagonized with flutamide, during the prenatal or postnatal periods in male rats, increased basal and stress induced concentrations of CORT are found in adulthood (Bingham and Viau, 2008; McCormick *et al*, 1998; McCormick and Mahoney, 1999; Seale *et al*, 2005a). Male rats treated perinatally

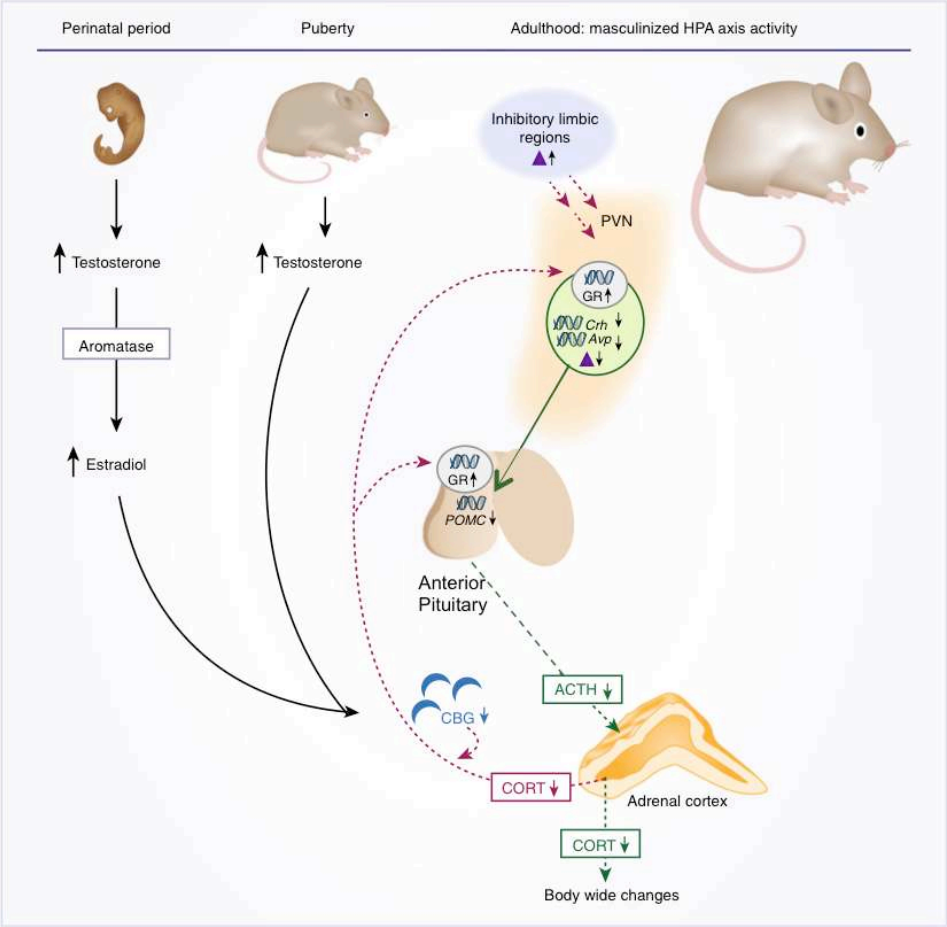

Figure 1.2. Organizational actions of gonadal hormones program lasting changes in the adult male hypothalamic pituitary adrenal (HPA) axis response to stress. Testosterone surges that occur during the perinatal period and puberty play important roles in masculinizing the HPA axis response to stress in adult male rodents. During the perinatal period, testosterone, largely via its conversion to estradiol by the aromatase enzyme, is involved in establishing a blueprint for a male typical pattern of HPA axis activity in adulthood. Pubertal testosterone then acts on this blueprint to complete the development of the masculinized HPA axis. Thus, in adulthood, males have decreased HPA axis responses to acute stressors characterized by decreased paraventricular nucleus (PVN) neuronal activation and gene expression, decreased pituitary expression of the proopiomelanocortin (POMC) precursor for adrenocorticotropin (ACTH), and decreased ACTH and corticosterone (CORT) responses to acute stressors. Adult males also have enhanced negative feedback resulting from their relatively reduced corticosteroid-binding globulin (CBG) levels, their increased PVN and pituitary glucocorticoid receptor (GR) gene expression, and their increased neuronal activation in limbic regions that inhibit the HPA axis. Organizational effects of testosterone, therefore, are important contributors to sex differences in the adult HPA axis in which females have relatively enhanced activity. Triangles indicate neuronal activation.

with flutamide also have altered patterns of basal CORT secretion as adults (Seale *et al*, 2005a). Normally, both male and female adult rodents secrete basal glucocorticoids in a pulsatile fashion throughout the day; but females have higher frequency and amplitude pulses than do males (Seale *et al*, 2004b). Perinatal AR antagonism in males increases their frequency and amplitude of CORT pulses in adulthood such that they resemble those of females. Notably, the effects of perinatal GDX on basal and stress enhanced glucocorticoid secretion in male rats are reversed by a single neonatal dose or neonatal doses of testosterone, but not prolonged testosterone treatment in adulthood (Bingham and Viau, 2008; McCormick *et al*, 1998). In females, a single neonatal dose of testosterone also dampens their HPA activity as adults (Seale *et al*, 2005b). Together, these findings indicate that neonatal testosterone acts through ARs to produce lasting masculinization of glucocorticoid secretion by the HPA axis.

In rodents, the conversion of testosterone to estradiol by the aromatase enzyme, and estradiol's subsequent actions in organizing brain function, set the stage for functional sex differences in the HPA axis in adulthood (Figure 1.2). Support for such estradiol-dependent sexual differentiation of the HPA axis comes from the identification of high levels of ERs (MacLusky *et al*, 1979) and aromatase enzymes (Shinoda *et al*, 1994) in the prenatal and neonatal rat brain. Additionally, treatment with an aromatase inhibitor or estradiol during the perinatal period increases and decreases basal and stress-induced HPA output in adult male rats, respectively (Bingham *et al*, 2012; McCormick *et al*, 1998; Seale *et al*, 2005a). Similarly, chronic estradiol treatment (estrogenization) of female neonates, results in an evening CORT rise in adulthood that resembles that of male rather than diestrous female rats (Patchev *et al*, 1995). Thus, both early androgen and estrogen actions through ARs or ERs can establish sex differences

in glucocorticoid secretion in adulthood, which are supported by sexually dimorphic activity at all levels of the HPA axis.

Current evidence supports a role for neonatal exposure to gonadal hormones in altering PVN neuroendocrine function during adulthood. GDX of male rats as neonates increases their basal and restraint-induced c-Fos expression (a measure of neuronal activation) in the medial parvocellular part of the PVN in adulthood (Bingham and Viau, 2008). These effects are reversed by neonatal, but not adult, treatment with testosterone, suggesting that neonatal testosterone contributes to male typical stress-induced PVN neuronal activation (Bingham and Viau, 2008). Furthermore, adult male rats treated with an aromatase inhibitor (1,4,6-androstatriene-3,17-dione (ATD)) as neonates have increased restraint-stimulated c-Fos mRNA in the PVN (Bingham *et al*, 2012). Collectively, these findings suggest that neonatal testosterone treatment, at least partially via its conversion to estradiol, contributes to male typical PVN neuronal activation following stress.

Early exposure to gonadal hormones also alters the expression of genes associated with HPA activation in adulthood. A single injection of testosterone in neonatal females is sufficient to decrease their stress-generated levels of PVN CRH, PVN AVP, and anterior pituitary POMC mRNAs as adults (Seale *et al*, 2005b). Additionally, estradiol treatment of female neonatal rats reduces basal PVN *Crh* gene expression in adulthood such that it resembles that of males rather than that of control diestrous females (Patchev *et al*, 1995). Such estrogenized females also do not exhibit increases in PVN CRH mRNA following adult OVX and estradiol replacement as do control females (Patchev *et al*, 1995). In males, on the other hand, perinatal exposure to either an AR antagonist (flutamide) or ATD markedly increases PVN CRH and anterior pituitary POMC mRNA following stress in adulthood (Seale *et al*, 2005a). Consequently, both testosterone and its

aromatization to estradiol are important for the development of masculinized HPA gene expression, which is reduced in adult males compared with females post-stress.

Limbic regulation of the HPA axis is also subject to organizational actions of gonadal steroids. Neonatal treatment with an aromatase inhibitor, ATD, increases neural activation in limbic brain regions known to regulate the HPA axis in adult males, such as the PFC, lateral septum, and MeA (Bingham *et al*, 2012). Moreover, neonatal GDX modifies expression of AR in the MeA and BNST, two areas that are important for regulating PVN neuronal activation and glucocorticoid secretion in response to stress (Bingham and Viau, 2008). Both stressed and unstressed adult male rats that were GDX'd as neonates have decreased numbers of AR expressing cells in both brain regions, and this effect is reversed by neonatal testosterone treatment (Bingham and Viau, 2008). Consequently, early testosterone exposure and aromatization enable the normal development of limbic structures that regulate the HPA axis in adult males.

Gonadal hormones similarly influence glucocorticoid negative feedback on the HPA axis, which is generally enhanced in males relative to females in adulthood. Accordingly GR expression is often greater in males than females in structures important for controlling the HPA axis, such as the hippocampus, PVN and pituitary (Karandrea *et al*, 2000, 2002; Turner and Weaver, 1985). However, this sex difference in adulthood likely depends on the estrous stage of the females examined, as one study found that adult diestrous females have greater hippocampal and PVN GR expression than males (Patchev *et al*, 1995). Nonetheless, early gonadal hormone exposure has been shown to modulate GR expression and contribute to its sex-biased expression in adulthood. One study found that neonatal estradiol treatment decreases adult levels of hippocampal GR mRNA such that they are indistinguishable from those in adult males,

suggesting that early estradiol exposure has "defeminizing" effects (Patchev *et al*, 1995). Furthermore, PVN GR mRNA is decreased, or "feminized", in adult male rats treated neonatally with either an AR antagonist or aromatase, whereas it is increased ("defeminized") in adult females treated neonatally with testosterone or estradiol (Patchev *et al*, 1995; Seale *et al*, 2005a, 2005b). In the anterior pituitary, adult males also have decreased GR binding if they were neonatally GDX'd (McCormick *et al*, 1998). Lastly, perinatal GDX elevates CBG levels in adult males (McCormick *et al*, 1998). Collectively, these findings suggest that the increased HPA negative feedback observed in adult males may be due, at least in part, to androgen-mediated effects via ARs and ERs on the epigenetic control of corticosteroid receptor expression and ligand availability.

Studies indicating sex differences in the necessity of forebrain and PVN GRs for HPA axis suppression may further support an organizational hypothesis of gonadal hormone action. These studies revealed that the loss of forebrain and PVN GRs results in dysregulation of HPA axis activity in males, but is largely inconsequential in females (Solomon *et al*, 2012, 2015). The use of randomly cycling females in these studies leaves open the possibility that activational effects of gonadal hormones influence GR involvement in HPA regulation. However, the substantial differences in HPA function between control females and those containing selective GR depletion- all of which were randomly cycling- would suggest that the effects of GR knockdown are not modified by estrous cycle stage. Rather, organizational effects of gonadal hormones may be at play and suggest that females use a different mechanism or neurocircuitry to inhibit HPA axis function.

Pubertal gonadal hormones and the HPA axis

Rises in gonadal steroids that occur at puberty provide another signal that can drive permanent changes in brain function. Although limited, some evidence exists that a second critical period for the organizational actions of gonadal hormones on the HPA axis may occur near puberty (Romeo, 2003). The increases in gonadal steroids during this period drive numerous neural and behavioral changes to produce adult-like characteristics (Romeo, 2003). These include changes in neuroendocrine function, such as the stress-induced activity of the HPA axis. Accordingly, rats of both sexes have greater glucocorticoid responses to acute stressors before puberty than they do in adulthood (Romeo *et al*, 2004b, 2004a). A thorough review of such age dependent changes in the HPA axis can be found in Romeo 2017 (Romeo, 2018).

Notably, neuroendocrine sex differences that begin to develop in the perinatal period are completed during puberty (Romeo, 2003). As is the case for testosterone exposure during the perinatal period, exposure during puberty is important for the masculinization of HPA responses to stress in adulthood (Goel and Bale, 2008). Adult female mice given testosterone during puberty have de(Goel and Bale, 2008). Moreover, pubertal testosterone exposure is important for organizing the adult male-typical sensitivity of the HPA axis to androgen regulation (Evuarherhe *et al*, 2009b). Unlike males GDX'd in adulthood, adult male rats GDX'd before puberty do not respond to testosterone administration with decreases in basal or stress-induced PVN gene expression or glucocorticoid secretion (Evuarherhe *et al*, 2009b). Thus, puberty is potentially a critical organizational period during which rising levels of androgens play a vital role in sculpting the correct development of a male-typical, androgen sensitive adult HPA phenotype.

Pubertal rises in estradiol may also play a role, although less pronounced, in the organization of the adult HPA axis. Prior to puberty, estradiol decreases the activity of the HPA

axis in female rats, whereas in adulthood it increases HPA activity (Evuarherhe *et al*, 2009a). Interestingly, this change in the HPA response to estrogens does not appear to depend on ovarian steroids (Evuarherhe *et al*, 2009a). In adult rats, estradiol treatment increases basal and stress induced glucocorticoid secretion regardless of whether they were OVX'd before or after puberty (Evuarherhe *et al*, 2009a). Thus, pubertal estrogen exposure is not essential for establishing the stimulatory effects of estradiol on the HPA axis in adult females.

Adolescence and the HPA axis

Adolescence marks a transitional period between weaning and adulthood characterized by significant brain development, including changes in the HPA axis (McCormick and Green, 2013). Although adolescence includes puberty and the associated effects of gonadal steroids on the HPA axis that occur during this period (discussed above), it can be more specifically divided into three stages: (1) prepubescence/early adolescence, (2) mid-adolescence, which includes pubertal onset and (3) late adolescence during which puberty terminates (McCormick and Green, 2013). Sex differences in HPA activity have been reported to emerge throughout adolescence. For example, adrenal volume increases in the late adolescence period more so in females than in males (Pignatelli *et al*, 2006). Sex biases also exist in the process whereby GRs translocate from the cytosol to the nucleus to influence gene transcription. One study demonstrated that GR translocation increases following acute stress in female, but not male, adolescent rats (Bourke *et al*, 2013). However, when adolescent rats have a history of chronic stress exposure, co-chaperones that inhibit GR translocation are upregulated and glucocorticoid negative feedback is impaired following an acute stressor only in females (Bourke *et al*, 2013). Additionally, gonadal hormones have been shown to play a role in regulating HPA function during adolescence, which can have lasting consequences. One study demonstrated that exposure to androgens irreversibly

masculinizes CBG concentrations in adulthood. Accordingly, adult males have increased levels of CBG (resembling those of females) if GDX'd during early adolescence (Mataradze *et al*, 1992). A thorough review of sex differences in the HPA axis during adolescence and the influence of gonadal hormones can be found in (Green and McCormick, 2016) and (McCormick and Mathews, 2007).

1.7 Remaining questions regarding sex differences in the HPA axis: effects of sex chromosomes and chronic stress

Do sex chromosomes affect the HPA axis?

Although activational and organizational actions of gonadal hormones are essential mediators of sex differences in neural and behavioral phenotypes, more recent evidence suggests that sex chromosomes can have direct effects in non-gonadal tissues that act alongside or even independent of gonadal hormone secretions (Arnold, 2009). Such chromosome effects partially involve expression of male- and female-specific genes by the Y and X chromosomes, respectively (see Arnold 2017 for review) (Arnold, 2017). For example, expression of the gene *Sry* by the Y chromosome in males not only causes differentiation of the testis, but can also act in the brain to alter its function (Arnold, 2017; Dewing *et al*, 2006). Thus, differences intrinsic to XX and XY cells may be key mediators of sex differences in diverse aspects of physiology and behavior, including the neuroendocrine response to stress (Arnold, 2017).

Unfortunately, studies examining the effects of XX versus XY on sex differences in the adult HPA axis are lacking. Nevertheless, rodent models now exist that would greatly aid in such investigations. The four core genotypes model has been used most frequently and involves the uncoupling of gonadal (ovaries versus testes) and chromosomal (XX versus XY) sex. Subjects accordingly include: (1) XX gonadal females (2) XY gonadal females, (3) XX gonadal males, and (4) XY gonadal males (see (Arnold and Chen, 2009) for review). Thus, the four core

genotypes allow for testing of the organizational/ activational effects of hormones resulting from gonadal sex independently of the intrinsic differences in XX versus XY cells (Arnold, 2009). This model has previously been used to examine sex differences in the morphology of AVP fibers in the lateral septum, a region known to modulate activity of the HPA axis. Results indicate that sexually dimorphic septal AVP not only arises due to organizational/activational effects of gonadal steroids, but also due to chromosomal effects (Gatewood, 2006; De Vries *et al*, 2002). Thus, it is highly likely that such sex chromosomal effects alter other aspects of HPA axis regulation, but this remains to be determined.

Are there sex differences in HPA axis following chronic stress?

Acute activation of the HPA axis by stress is considered an adaptive response to the increased metabolic demand required to deal with the stressor. Yet, in the face of chronic stress, this same response can eventually be considered maladaptive and lead to greater risk for disease states (De Kloet *et al*, 2005). A particularly well-established association exists between hyperactivity of the HPA axis and the presentation of affective disorders, such as depression and anxiety (Bangasser and Valentino, 2014; Martin *et al*, 2010). Such disorders are of notable interest since they exhibit a striking sex bias in prevalence. Women are at least twice as likely as men to present with these conditions, and this bias is likely driven (at least in part) by sex differences in the physiological response to chronic stress (Bangasser and Valentino, 2014). Thus, some studies have begun to examine sex differences in the activity of the HPA axis under chronic stress conditions in the interest of understanding why women have increased vulnerability to affective disorders.

Models frequently used to examine HPA dysregulation that may predispose or trigger stress-related diseases are the repeated (homotypic) stress and the chronic mild stress (CMS)/

chronic variable stress (CVS) models. The repeated stress model involves presentation of the same stimulus continually until a reduction in the physiological response, or "habituation", to that stressor occurs (Dhabhar *et al*, 1997). The CMS/CVS model, alternatively, prevents habituation of the stress responses by exposing animals to randomly presented stressors over a prolonged period (Willner, 2017). Both models are thought to be etiologically relevant for human disease risk and have been used to examine sex differences in the HPA axis response to chronic stress, as discussed below.

Sex differences following repeated stress

After a number of exposures to the same stressor, sex differences are present in the basal activity of the HPA axis in which female rats have higher plasma CORT and CBG levels than males (Galea *et al*, 1997; Vieira *et al*, 2018). Additionally, sex differences may be found in the habituation of HPA activity in repeated stress scenarios. In both sexes, this habituation is characterized by decreased ACTH and CORT responses to a stressor, as well as reduced stress-induced neuronal activation in the medial parvocellular part of the PVN, as the number of exposures increases (Zavala *et al*, 2011). However, the degree to which the habituation of HPA activity occurs in male and female rodents may vary. Some studies report similar habituation between the sexes. Accordingly, CORT responses to restraint or loud noise stressors incrementally decrease by the same amount in male and female rats (Babb *et al*, 2014). The number of activated, c-Fos expressing neurons that co-express AVP following restraint stress also similarly decreases in males and female rats (Zavala *et al*, 2011). Other studies, alternatively, suggest that habituation in a repeated restraint stress paradigm is reduced or slower in female versus male rats (Bhatnagar *et al*, 2005; Chadda and Devaud, 2005; Galea *et al*, 1997).

Notably, this sex difference has also been shown to be accompanied by a decrease in plasma CBG levels only in female rats (Galea *et al*, 1997).

Morphological changes may also accompany chronic stressors and these can also show sex biases. Whereas repeatedly restrained males exhibit apical dendritic atrophy of CA3c pyramidal neurons in the hippocampus, females show a decrease in basal dendritic branching (Galea *et al*, 1997). Ultimately, whether or not habituation is sex-biased likely depends on the type of stressor and/ or experimental paradigm. Nonetheless, findings to date largely support the possibility that females have enhanced HPA axis responses during repeated stress, which may contribute to their increased susceptibility to stress-related diseases.

Some studies have also examined sex differences in facilitation, a process that occurs when repeatedly stressed subjects have an equal or greater response to a novel stressor than control subjects (Dallman and Jones, 1973). When exposed to a novel acute restraint stress, chronically cold-stressed male mice exhibit similar ACTH and CORT responses to control males (Chen and Herbert, 1995). Cold-stressed females, on the other hand, have lower ACTH responses than, but similar CORT responses to, unstressed control females following a novel stressor (Chen and Herbert, 1995). These findings suggest that both sexes show facilitation, but that it may be more prominent in males. Conversely, another study found no sex differences in the HPA axis response to a novel environment following repeated exposure to either audiogenic or restraint stress (Babb *et al*, 2014). Together, these studies suggest that sex differences in facilitation are stressor dependent.

The influence of gonadal hormones on HPA responses to repeated stressors has also been investigated. During repeated restraint stress, estradiol has been shown to limit habituation. Thus, in OVX female rats treated with estradiol, the decrease in plasma CORT responses following

repeated stress exposure is lesser than it is in vehicle-treated controls (Lunga and Herbert, 2004). In the same estradiol-treated OVX females, PVN CRF and AVP mRNA levels are also enhanced by chronic stress, and the habituation of AVP mRNA is impaired (Lunga and Herbert, 2004). These findings suggest that, like in acute stress studies, estradiol increases HPA responses to repeated stress in female rats. Conversely, estradiol has been shown to dampen the effects of repeated footshock stress on activity in the PVN (Gerrits *et al*, 2005). Accordingly, in OVX female rats given estradiol, c-Fos expression is lesser in the PVN following repeated stress than in vehicle-treated controls (Gerrits *et al*, 2005). The differential effects of estradiol on the repeated stress-induced activity of the HPA axis may be due to varying experimental parameters, such as the type of stressor or the treatment duration. However, it is also possible that estradiol has varying effects depending on whether ERα- or ERβ-mediated mechanisms are involved, as discussed above for acute stress situations. In support of this possibility, the study by Gerrits et al. 2005 demonstrated that estradiol treatment increases the number of ERβ-expressing cells in the PVN observed during chronic footshock stress, supporting ERβ mediated mechanisms of PVN neuronal inhibition. Regardless of the direction of influence, the studies presented here demonstrate an important role for estradiol in determining how females adapt to chronic stress, which may influence sex differences in stress-related pathology.

The effect of gonadal hormones on chronic stress-induced HPA activity has also been investigated in male subjects. In male rats exposed to repeated restraint stress, GDX enhances the habituation of the plasma CORT response to the restraint. However, habituation of the neuronal c-Fos response to repeated restraint stress in the PVN, MeA and locus coeruleus are not affected by GDX (Chen and Herbert, 1995). Therefore, androgens may minimally decrease HPA activity during repeated stress, but further study is necessary to fully determine this. Another study

demonstrated that testosterone has organizational rather than activational effects that influence the habituation of the HPA axis during repeated psychogenic stress in male rats (Bingham *et al*, 2011a). Accordingly, males treated with an androgen receptor antagonist (flutamide) or an aromatase inhibitor (ATD) during the perinatal period have a reduced decline in ACTH and CORT responses to repeated stress in adulthood. When adult males are GDX'd, on the other hand, no changes in the habituation of the CORT response to stress are evident (Bingham *et al*, 2011a). Thus, both the activation of ARs and the conversion of testosterone to estradiol during the perinatal period- but not androgen exposure during adulthood- are essential for males to exhibit habituation during adulthood (Bingham *et al*, 2011a).

Sex differences following chronic mild or variable stress

Studies using the CMS and CVS models in male rodents have revealed that chronic stress generally enhances HPA axis activity, as indicated by increased basal CORT secretion, increased PVN CRH and AVP mRNA, and decreased GR expression in inhibitory brain regions (Dallman, 1993; Herman, 2013). Although some studies have begun to compare HPA activity following CMS/CVS in male and female rodents, these are relatively few in number.

HPA axis dysregulation has been shown to be greater following CMS and CVS in female versus male rats, as females have larger elevations in basal CORT secretion than males post CMS and CVS (Dalla *et al*, 2005; Vieira *et al*, 2018; Xing *et al*, 2013). Yet, chronically stressed males do exhibit adrenal hypertrophy and have elevated CORT levels when faced with a novel stressor, whereas chronically stressed females respond similarly to control, non-CMS females (Dalla *et al*, 2005; Vieira *et al*, 2018). This suggests that in males, but not females, a chronic stress history results in a potentiated HPA axis response to a novel stressor. Thus, females may be more vulnerable than males to chronic stress in the first place, but they utilize different or

better coping skills when faced with a novel stressor. Whether these results indicate that one sex is better or worse off cannot be determined without more information. Females may adapt better in a CMS scenario, which may be important for stress resilience. However, if an acute response to a novel stressor following CMS is beneficial, females may be relatively impaired.

Sex differences in PVN neuronal activity following CMS have also been examined. In CMS exposed rats, c-Fos expression is increased in the PVN of socially-housed males, but not socially-housed females (Westenbroek *et al*, 2003a). Yet, both males and females have increased PVN c-Fos expression following CMS when singly housed (Westenbroek *et al*, 2003a). These findings suggest that social support for females may enable them to better cope with stress and is beneficial, whereas social housing in males may not be beneficial and may even exacerbate their vulnerability to stress (Westenbroek *et al*, 2003a). They support the possibility that social environment modifies the activity of the HPA axis (Kirschbaum *et al*, 1995). Additionally, sex differences in PVN gene expression changes have been observed following CMS (Duncko *et al*, 2001). Constitutive PVN *Crh* gene expression is increased in males, but not females, post CMS which may be due to the females' increased basal PVN CRH mRNA levels relative to those of males (Duncko *et al*, 2001). Accordingly, males may require greater increases in CRH from their lower basal levels to meet the demands of CMS. It is also possible that females have greater increases in *Avp* or *Ot* gene expression to make up for their lower CRH levels post CMS; or females could have CBG and/or MR levels that result in decreased glucocorticoid negative feedback. Although these possibilities require further investigation, the findings of this study and that showing sex differences in PVN neuronal activation support the possibility that HPA axis activity post CMS is increased in males and socially isolated, but not group housed, females.

Future directions for chronic stress studies

Further examination of the HPA axis response to chronic stress in both sexes is necessary to better assess sex differences. Although studies have begun to examine sex differences in PVN gene expression and neuronal activation following repeated stress and CMS, questions remain regarding sex differences at other levels of HPA axis regulation. For instance, are there sex differences in the limbic and brainstem structures that control the activity of the HPA axis following chronic stress? One study suggests that female, but not male rats, have deceased serotonergic activity in the hippocampus after CMS (Dalla *et al*, 2005). This difference may underlie the increased vulnerability of females to CMS, but it remains unclear how other regulatory brain regions may be affected to further contribute to this sensitivity. Similarly, following repeated stress, sex-biased changes in the morphology of hippocampal neurons have been observed, however investigation of changes in other regulatory brain regions is lacking (Galea *et al*, 1997). Additionally, whether or not sex differences exist in *Ot* and *Avp* gene expression in the PVN and/or in POMC expression in the anterior pituitary remains to be better determined using both chronic stress models. Sex differences in adrenal sensitivity to ACTH and glucocorticoid feedback regulation are similarly under-investigated. One study did examine the role of the PVN GR in the regulation of chronic stress reactivity, but found that selective deletion of GR in the PVN has no effect on the CORT response to CMS in either male or female mice (Solomon *et al*, 2015). This finding suggests that glucocorticoid control of the HPA axis after CMS likely does not involve the PVN and raises questions about whether other limbic and brainstem regions and/or the anterior pituitary serve as compensatory targets for glucocorticoid negative feedback in a sex-dependent manner. In a repeated stress model, alternatively, females show greater numbers of c-Fos and GR positive PVN cells than males, suggesting that females

may be more sensitive to glucocorticoid negative feedback in this case, but further investigation is necessary (Zavala *et al*, 2011). Furthermore, in both chronic stress models, sex differences in the mechanisms used by the GR and MR to negatively regulate the activity of the HPA axis have not been examined. Sex differences in receptor activation, assembly, or transactivation, for instance, may be present. Ultimately, it will be important to investigate sex differences at all levels of HPA axis regulation in response to repeated and variable chronic stressors moving forward. Such investigations will provide a more complete picture of the neurobiological mechanisms that coordinate to produce sex differences in vulnerability and resilience to chronic stress.

Moreover, few, if any, studies have examined the influence of gonadal hormones on sex-dependent HPA activity following CMS/CVS. The corresponding studies following repeated stress are greater in number, but still limited. This leaves questions regarding whether changes at each level of HPA axis regulation are influenced by the organizational and/or activational actions of gonadal hormones as has been demonstrated in acute stress studies. There are also especially large gaps in our understanding of how gonadal steroids may come to program lasting sex differences in the HPA axis response to stress in females (Viau and Innala, 2016). Although further investigation is necessary, the rodent literature certainly supports the possibility that gonadal hormones alter the chronic stress induced activity of the HPA axis in both sexes. For one, chronic stress is well associated with a dysregulation of the HPG axis, which involves inhibition of estrogen and testosterone secretion, as well as desynchronization of estrous cycling in females (Dalla *et al*, 2005) (see (Panagiotakopoulos and Neigh, 2014) and (Joseph and Whirledge, 2017) for review). Thus, it is likely that chronic stress disrupts the HPG axis to contribute to the dysregulation of the HPA axis classically found following chronic stress

(Swaab *et al*, 2005). Additionally, endogenous gonadal hormone levels may have a profound influence on HPA activity when altered by chronic stress exposure during critical periods. Early life stress, for instance, can disrupt key organizing actions of gonadal hormones that masculinize the HPA axis and produce lasting effects on rodents' vulnerability to chronic stress in the future (Bale and Epperson, 2015). Similarly, the activational action of gonadal hormones can be disturbed by chronic stress in adulthood (Bale and Epperson, 2015). Ultimately, understanding how gonadal hormones modulate the HPA axis response to chronic stress has important implications for understanding the pathology of affective disorders, which are associated with major changes in gonadal hormone levels (see (Mueller *et al*, 2014) for review).

1.8 Sex differences in the HPA axis and disease in humans

Understanding sex differences in the human HPA axis has been a topic of much interest, as dysregulation of the HPA axis (hypo- or hyper- reactivity) is a hallmark of many stress-related diseases, which are known to differentially present in men versus women (Bangasser and Valentino, 2014). In humans, as in rodents, sex differences exist in the HPA axis response to stress and are largely age- and modality-dependent, although findings are not always consistent between the species. Before puberty, male and female rodents exhibit limited differences in their physiological stress response, as do boys and girls (Kudielka and Kirschbaum, 2005; Romeo, 2010). However, during adolescence and into adulthood, male rodents begin to show a blunted HPA axis response to stress relative to females and human findings are much less consistent (Bangasser and Valentino, 2014; Gomez *et al*, 2004). While some studies suggest that adult women have enhanced HPA responses to acute stressors compared to males, others indicate greater responses in males or no significant difference (Bangasser and Valentino, 2014; Kirschbaum *et al*, 1999; Kudielka and Kirschbaum, 2005; Seeman *et al*, 2001; Uhart *et al*, 2006).

These contradicting findings could be due to a number of factors, with the type of stressor being important, as well as the age, overall health and menstrual cycle stage of the female participants (Bangasser and Valentino, 2014; Kirschbaum *et al*, 1999; Seeman *et al*, 2001). For a more thorough review of sex differences in the human HPA axis, please see Bangasser and Valentino, 2014 and Kudielka and Kirschbaum 2005.

Organizational/ activational effects of gonadal hormones also influence sex differences in the human HPA axis as they do in rodents. Studies examining females in varying stages of the menstrual cycle, compared with males, have accordingly found that sex differences in the salivary cortisol response to psychosocial stress are present when women are in the low-estrogen state of the follicular phase but not the lueteal phase (Rohleder *et al*, 2001). This suggests a role for estrogen and progesterone in regulating stress responsiveness in women across the menstrual cycle. Moreover, support for a role of gonadal hormones comes from the sex-biased prevalence of stress-related diseases, such as major depressive disorder, which develops after after puberty (Bale and Epperson, 2015).

During early gestation, exposure to maternal and/or environmental stress produces more adverse outcomes in male than female offspring (Bale and Epperson, 2015; Sandman *et al*, 2013). Accordingly, neurodevelopmental disorders, such as autism spectrum disorder and attention deficit hyperactivity disorder, are more common in boys than girls (Erksine *et al*, 2013; Newschaffer *et al*, 2007). This discrepancy potentially reflects a failure of neonatal testosterone to prepare the brain in early life for adult levels of testosterone, which complete the masculinization of the brain during puberty (See Bale and Epperson, 2015 for review). During adolescence and throughout adulthood, however, females have increased risk for affective disorders, such as depression and anxiety, especially if they have experienced early life adversity

(Bangasser and Valentino, 2014; Heim and Nemeroff, 1999). Such findings are also consistent with the greater risk for comorbidity of major depressive disorder with cardiometabolic diseases in women, which may be related to sex-biased HPA activity, as well as sex-biased activity in the autonomic nervous system in which females have enhanced parasympathetic tone (Goldstein *et al*, 2011, 2014). Collectively, these clinical findings indicate that the timing of developmental perturbations can have long-term sex-biased outcomes; and they support a role for adult levels of female gonadal hormones in increasing risk for stress-related diseases (See Bale and Epperson, 2015 for review). Ultimately, prior to starting any therapy for stress-related diseases, clinicians should be aware of sex differences in HPA axis activity and their underlying sources.

1.9 Conclusions

Striking sex differences exist in the HPA response to acute stress and its underlying circuitry in rodents. Generally, females exhibit enhanced glucocorticoid secretion in response to various acute stressors, which is supported by evidence of sex differences at all levels of HPA axis regulation (Handa and Weiser, 2014). Abundant evidence also supports organizational and activational roles for gonadal hormones in driving sex differences in acute HPA axis activity (Green and McCormick, 2016). However, further study is necessary to rule out the possibility of sex chromosome effects that act with or without gonadal steroids to modulate HPA activity. Moreover, sex differences in the HPA axis after chronic stress remain largely under-investigated. Chronic rather than acute stress results in the dysregulation of the HPA axis that may increase risk for stress-related diseases in humans (De Kloet *et al*, 2005). Thus, investigations of sex differences in chronic stress-induced HPA dysregulation and the underlying influences of gonadal hormones and sex chromosomes may have important therapeutic consequences in the future.

1.10 Addendum: Steroid hormone receptor structure and function

Steroid hormone receptors (SHRs), comprising subfamily 3 of the nuclear receptor superfamily, are intracellular proteins that monitor steroid hormones freely permeable to the cell membrane (Nuclear Receptors Nomenclature Committee, 1999). Together the estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR) and estrogen-related receptor, make up this subfamily (Nuclear Receptors Nomenclature Committee, 1999). These receptors, like others in the nuclear receptor superfamily, exhibit conserved characteristics. They typically have a low copy number per cell and high affinity for their ligand (Beato and Klug, 2000). They also can exist in multiple forms (Hollenberg *et al*, 1985; Kastner *et al*, 1990; Kuiper *et al*, 1996). Through their function as ligand-activated transcriptional regulators, SHRs ultimately provide a direct link between physiological signaling processes and transcriptional responses (Evans, 1988).

The classic model of SHR action posits that a SHR in the cytoplasm or nucleus interacts with chaperone proteins, which are dissociated upon ligand binding due to a conformational change in the receptor. The ligand-activated receptor then dimerizes and translocates to chromatin DNA where it can directly influence gene transcription (Giguère, 2010) (Figure 1.3a). Characteristic modules based on conserved regions and functions facilitate this classic SHR-mediated signaling, with an A/B domain, DNA binding domain, and ligand binding domain (LBD) playing especially predominant roles (Giguère *et al*, 1986; Hollenberg *et al*, 1987; Hollenberg and Evans, 1988; Jenster *et al*, 1991). For a thorough review of the individual functions of each domain see (Beato and Klug, 2000; Klinge, 2018; Kumar and Litwack, 2009; Rhen and Cidlowski, 2009). Briefly, the A/B domain is localized at the N-terminal end of the receptor protein, varies highly in sequence among nuclear receptors, and is primarily responsible

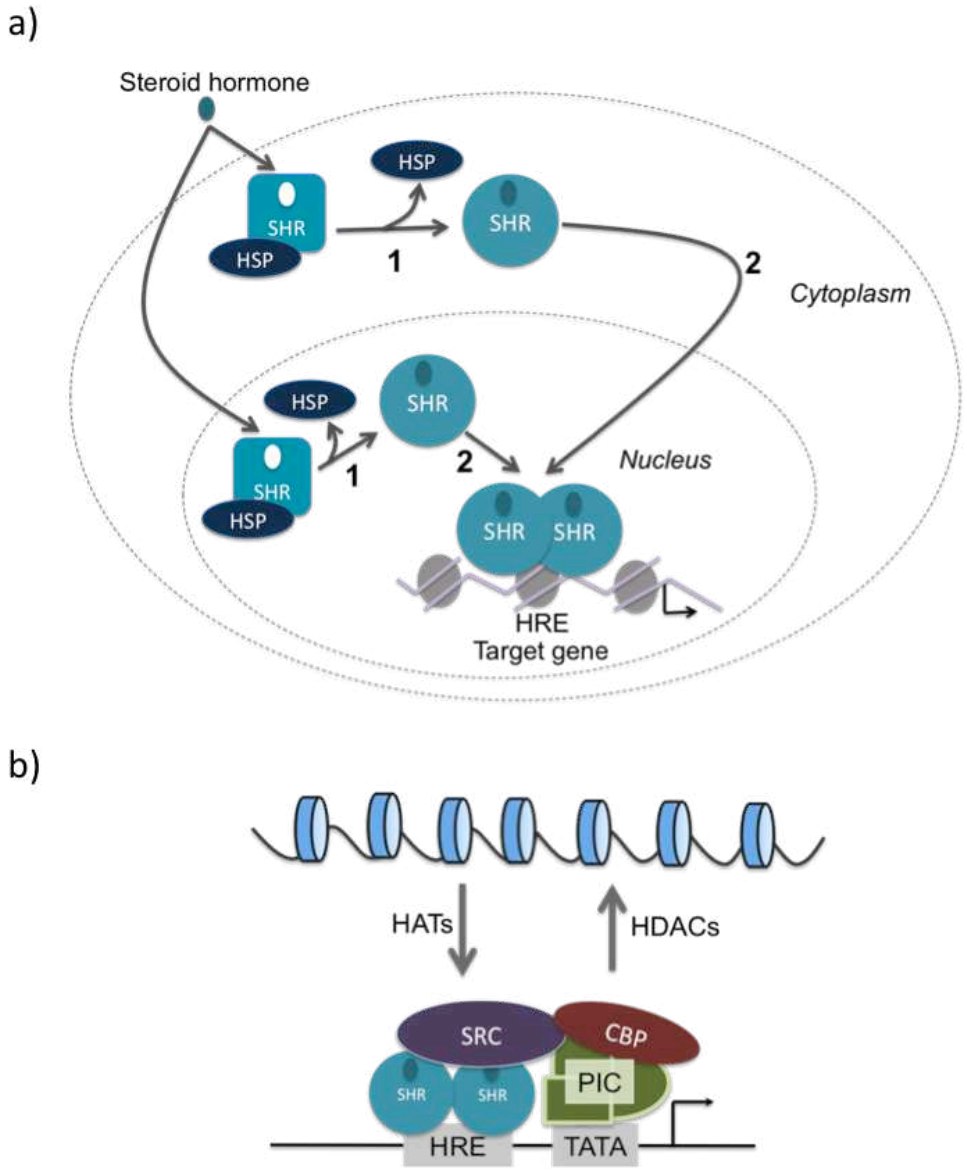

Figure 1.3. Classical model of steroid hormone receptor (SHR) signaling. A steroid hormone freely defuses through the plasma membrane where it can bind to a specific cytoplasmic or nuclear SHR, which is stabilized by heat shock protein (HSP) (a). Binding of the steroid hormone triggers a conformational change in the SHR, leading to the release of HSP (a; 1). The SHR then translocates to chromatin DNA where it binds a hormone response element (HRE) as part of a homo- or heterodimer (a; 2). SHR dimers can recruit co-regulatory proteins that have intrinsic histone acetyl transferase (HAT) activity or histone de-acetylase (HDAC) activity (b). Co-activator proteins, such as steroid receptor coactivator (SRC) and cAMP response element binding protein binding protein (CBP) have HAT activity and can unwind DNA from histones to facilitate the access of the transcriptional pre-initiation complex (PIC) to the transcription start site (black arrow). They also can activate the PIC. HDACs, alternatively, inhibit transcription by inducing chromatin recoiling. TATA, core promoter sequence.

for the receptor's trans-activation ability. The DNA binding domain is the most conserved region of the receptor and is composed of two zinc fingers that directly bind to DNA. Some degree of selectivity of the receptor for DNA is conferred by a varying series of amino acids within the knuckle of the first zinc finger (i.e. c**EG**ck**A** for ERs versus c**GS**ck**V** for GRs). The LBD confers the essential property of specific hormone recognition and can be thought of as a molecular switch that shifts the receptor from a transcriptionally silent to active state. Upon ligand binding to a large hydrophobic pocket that comprises the LBD, a conformational change in the receptor occurs. This transformation hides a heat shock protein (HSP)-binding interface, exposes a dimerization motif, and exposes binding surfaces for transcriptional co-regulators. Hiding of the HSP binding interface specifically leads to the dissociation of HSP 90, which allows for the exposure of domains enabling, for example, nuclear localization and DNA binding.

To bind DNA and directly influence gene transcription, SHRs bind DNA as monomers (Johnston *et al*, 1997), homodimers (Vanacker *et al*, 1999) or heterodimers (Pettersson *et al*, 1997) at specific sites termed hormone response elements (HREs) (Beato and Klug, 2000). The PR, GR, MR and AR all bind to similar HREs, originally described as glucocorticoid response elements (GREs) (Denayer *et al*, 2010; Karin *et al*, 1984; Scheidereit *et al*, 1983; Von Der Ahe *et al*, 1985), though not necessarily with the same degree of interaction (Beato, 1989; Cato *et al*, 1988; Chalepakis *et al*, 1988; von Der Ahe *et al*, 1986). ERs and estrogen related receptors, alternatively, bind estrogen response elements (EREs) and estrogen-related response elements (ERREs), respectively (Beato and Klug, 2000). For nuclear receptor subfamily 3 specifically, these HREs are characterized by a 15 nucleotide consensus sequence, including a six nucleotide palindromic sequence separated by three variable nucleotides (e.g. AGAACAnnnTGTTCT for the GRE where n= a variable nucleotide) (Rhen and Cidlowski, 2009). Because of the helix

formation of DNA, both parts of the palindromic sequence are brought together on the same side of a DNA molecule, such that the two DBDs of the receptor dimer each bind an HRE half site. Notably, transcriptional regulation by SHRs does not always require binding to a HRE. Both ER and GR have been shown to influence gene expression through their associations with activator protein-1 (AP-1) proteins, including Fos and Jun (Chinenov and Kerppola, 2001; Pak *et al*, 2005; Pearce *et al*, 1998).

To regulate transcription, one possibility is that ligand activated SHRs interact directly with the pre-initiation complex (Beato and Klug, 2000) (Figure 1.3b). This complex contains general transcription factors that are important for promoting polymerase II activity at the transcription initiation site (Kim *et al*, 1997). Additionally, SHRs regulate transcription via their recruitment of co-regulatory proteins that influence the accessibility of target promoters to the pre-initiation complex (Beato and Klug, 2000) (Figure 1.3b). To alter such accessibility, SHR co-regulatory proteins add or remove acetyl groups from histone proteins around which DNA is tightly coiled in a transcriptionally inactive state (Monroe and Spelsberg, 2004). The addition of acetyl groups, acetylation, unwraps DNA from histones, enabling the pre-initiation complex to access DNA sites, whereas the removal of acetyl groups, de-acetylation, acts in opposition (Bolger *et al*, 2007). Thus, steroid hormone receptor co-activator proteins often have histone acetyl transferase activity (HAT) or attract other proteins with HAT activity to activate transcription (Monroe and Spelsberg, 2004). Examples include steroid receptor coactivators (SRC) 1, 2 and 3, and cAMP response element binding protein (CREB) binding protein (CBP) which have HAT activity (Ogryzko *et al*, 1996; Spencer *et al*, 1997), as well as CREB which recruits CBP (Robyr *et al*, 2000). Steroid hormone receptor co-repressor proteins, alternatively, have histone de-acetylase (HDAC) activity or attract other proteins with HDAC activity to

repress transcription (Monroe and Spelsberg, 2004). For example, HDACs 1, 2 and 3 have HDAC activity, while nuclear co-repressor 1 recruits HDACs to DNA promoter regions (Robyr *et al*, 2000). The activity of co-regulators depends partly on the their expression levels in specific cell types; and the composition of the receptor/co-regulator complex largely defines the transcriptional potential of the SHR (Kumar and Litwack, 2009).

1.11 Addendum: Corticotropin releasing hormone (CRH) in the paraventricular nucleus (PVN) as an important target of glucocorticoid negative feedback[2]

Before the biochemical characterization of CRH (Vale *et al*, 1981), Oxytocin (OT) and arginine vasopressin (AVP) were also known to have CRH properties (Gibbs, 1986). Accordingly, while CRH is the most potent adrenocorticotropin (ACTH) secretagogue, AVP and OT also play roles in ACTH synthesis and secretion by the anterior pituitary (Gibbs, 1986). AVP's CRH-like properties have been particularly well studied. Although AVP is synthesized by and secreted from magnocellular neurons in the PVN that are involved in regulating extracellular fluid volume among numerous other physiological and behavioral functions, AVP is also produced by and released from parvocellular PVN neurons where it functions in regulating the hypothalamic pituitary adrenal (HPA) axis (Albers, 2012; Ludwig, 1995; McCann *et al*, 2003). In humans and rats, AVP is co-expressed in some parvocellular CRH neurons (Sawchenko *et al*, 1984; Tramu *et al*, 1983), and it strongly potentiates the ACTH secretagogue effect of CRH (Hauger and Aguilera, 1993; Torpy *et al*, 1994).

To down-regulate the stress-induced activity of the HPA axis, glucocorticoids can decrease either *Crh* or *Avp* gene expression in the PVN. Accordingly, most rat parvocellular

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[2]Addendum 1.11 is modified from: Heck AL, Crestani CC, Fernandez-Guasti A, Larco DO, Mayerhofer A, Roselli CE. Neuropeptide and steroid hormone mediators of neuroendocrine regulation. Journal of Neuroendocrinology. Vol. 30, No. 10, e12599, April 12, 2018. © British Society for Neuroendocrinology

PVN AVP and CRH neurons co-express glucocorticoid receptors (GRs), which translocate to the nucleus in the presence of a GR ligand, such as dexamethasone (Uht *et al*, 1988). Additionally, increases in both parvocellular PVN CRH and AVP heterogeneous nuclear RNA (Kovács *et al*, 2000; Ma and Aguilera, 1999), mRNA (Davis *et al*, 1986; Ma and Aguilera, 1999; Viau *et al*, 1999), and protein (Kovács *et al*, 1986; Sawchenko, 1987a, 1987b) levels have been reported in male rodents whose endogenous glucocorticoid production is removed by adrenalectomy (ADX). Peripheral administration of corticosterone (CORT) also inhibits medial parvocellular CRH and AVP mRNA responses to ADX in male rats (Viau *et al*, 1999). Similarly, in humans, suppressed hypothalamic CRH and AVP protein levels are evident following corticosteroid-exposure (Erkut *et al*, 1998).

Although CRH and AVP are both targets of glucocorticoid negative feedback, an especially predominant role has been identified for PVN CRH. In GR-deficient mice, CRH, but not AVP, peptide levels in the median eminence are substantially elevated compared to that of wild type controls (Kretz *et al*, 1999). Thus, at least at the hypothalamic level, CRH synthesis is the major target for feedback control and AVP synthesis may play a more cooperative role. Supporting this possibility, mice with central nervous system-specific knockout of GR exhibit increased PVN CRH protein, whereas PVN AVP expression remains unchanged (Tronche *et al*, 1999). However, other studies do suggest that PVN *Avp* gene expression may be more sensitive to glucocorticoid negative feedback than that of *Crh* (Kovács *et al*, 2000; Ma *et al*, 1997a; Makino *et al*, 1995b).

Mechanisms of GR repression of PVN *Crh*

While the GR can influence PVN *Crh* expression through actions in limbic areas important for regulating the HPA axis (Herman *et al*, 2016), this dissertation primarily explores

direct actions of the GR in PVN CRH neurons during delayed glucocorticoid negative feedback (minutes to hours) (Dallman *et al*, 1987). Abundant evidence supports such direct actions. Implants of the GR agonist, dexamethasone, near the PVN inhibit CRH protein expression in parvocellular neurons of the PVN in adrenalectomized (ADX'd) male rats (Kovács *et al*, 1986; Kovács and Mezey, 1987; Sawchenko, 1987a). Similarly, GR agonists directly inhibit basal and cAMP-induced CRH mRNA expression in slice cultures of the rat hypothalamic PVN lacking extra hypothalamic connections (Bali *et al*, 2008). In mice with defective GR function localized to the PVN, enhanced PVN CRH protein (Jeanneteau *et al*, 2012) and mRNA levels (Laryea *et al*, 2013) are also observed. Furthermore, GR immunoreactivity has been identified in parvoceullar CRH neurons of the ADX'd rat PVN (Uht *et al*, 1988); and a recent single cell RNA-sequencing analysis demonstrated that PVN CRH neurons express GR mRNA (Romanov *et al*, 2015).

During delayed glucocorticoid negative feedback on PVN *Crh*, the GR functions as a nuclear receptor and, prior to ligand binding, exists as part of a cytoplasmic multiprotein complex (Brink *et al*, 1992; de Kloet *et al*, 1998). This complex includes chaperone proteins (heat shock protein (hsp) 70, hsp90, and p23) as well as immunophilins of the FK506 family (FKBP51 and FKBP52) that stabilize the receptor in a conformation that is transcriptionally inactive but favors high affinity ligand binding (Oakley and Cidlowski, 2013). Upon binding glucocorticoids, the GR undergoes a conformational change resulting in the dissociation of connected proteins, multiple phosphorylation steps, nuclear translocation, dimerization, and ultimately the increased affinity of the receptor for DNA elements where it can influence gene transcription by recruiting transcriptional co-regulators (Brink *et al*, 1992; de Kloet *et al*, 1998). Whereas the *Crh* proximal promoter does not contain a consensus glucocorticoid response

element (GRE), it does contain a putative negative GRE (nGRE), which binds a monomer of the GR with high affinity and is thought to mediate glucocorticoid repression of *Crh* transcription (Malkoski *et al*, 1997; Malkoski and Dorin, 1999). It is still debated whether GR-mediated repression of *Crh* occurs via direct binding to the nGRE, or through inhibition of the activity of a nearby cAMP response element (CRE), which plays an important role in phosphorylated cAMP response element binding protein (pCREB) activation of *Crh* transcription (Guardiola-Diaz *et al*, 1996; Malkoski *et al*, 1997; Malkoski and Dorin, 1999; Yamamori *et al*, 2007). Evidence supporting each of these possibilities is discussed below.

Down-regulation of CRE-induced *Crh* expression

Activation of *Crh* transcription depends on the CRE's recruitment of cAMP response element binding protein (CREB) following its phosyphorylation by a large number of signaling pathways, including cAMP/ protein kinase A dependent ones (Seasholtz *et al*, 1988). Thus, to inhibit *Crh* expression, some studies suggest that glucocorticoids can oppose *Crh* induction via the CRE. Treatment with the GR agonist dexamethasone has been shown to inhibit stress-induced phosphorylation of CREB in parvocellular CRH neurons of the rat PVN, suggesting that glucocorticoids may interfere with CREB binding to the CRE in order to down regulate *Crh* expression (Légrádi *et al*, 1997). Additionally, heterologous promoter studies in cultured cells suggest that putative GR binding sites in the *Crh* promoter do not solely mediate its negative regulation by glucocorticoids (Guardiola-Diaz *et al*, 1996). Rather, the CRE appears to be required for cAMP-stimulated CRH reporter expression, as well as glucocorticoid repression (Guardiola-Diaz *et al*, 1996). Furthermore, GR signaling has been suggested to limit *Crh* expression by opposing the excitatory influence of brain derived neurotrophic factor (BDNF), which involves tropomyosin receptor kinase B (TrkB)-CREB signaling (Figure 1.4)

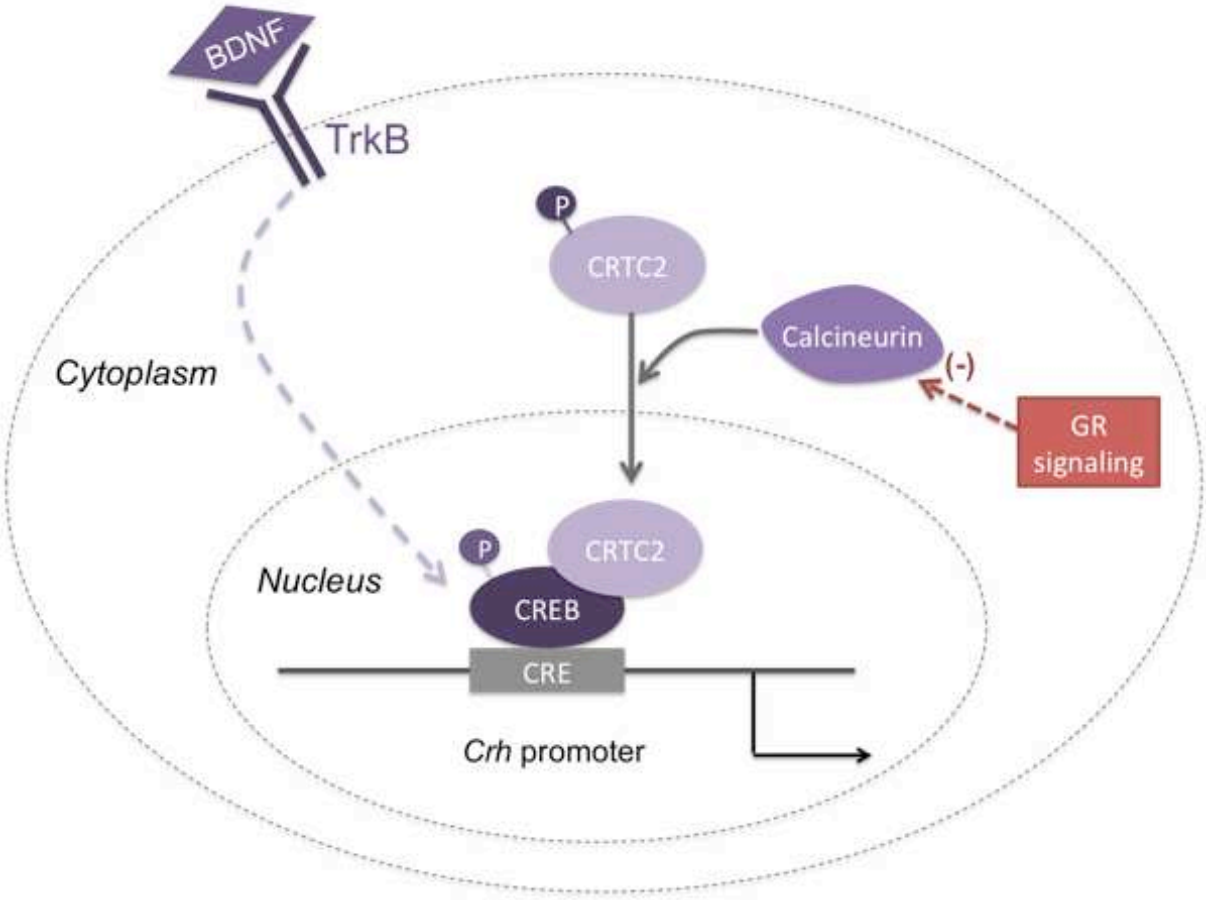

Figure 1.4. Proposed mechanism of glucocorticoid receptor (GR) inhibition of corticotropin releasing hormone (*Crh)* activation by brain derived neurotrophic factor (BDNF) signaling. BDNF, via activation of its receptor tropomyosin receptor kinase B (TrkB), leads to increased phosphorylated cAMP response element binding protein (pCREB), which is recruited to the cAMP response element (CRE) of the proximal *Crh* promoter to increase transcription. The CREB regulated transcription coactivator 2 (CRTC2) must be dephosphorylated in order to translocate to the nucleus where it cooperates with pCREB to regulate *Crh* expression. GR signaling may inhibit the activity of the phosphatase calcineurin to sequester CRTC2 in the cytoplasm and impair pCREB induced *Crh* transcription.

(Jeanneteau *et al*, 2012). Specifically, GR signaling is thought to deactivate CREB-mediated *Crh* induction by neutralizing the function of the CREB regulated transcription coactivator 2 (CRTC2). CRTC2 must be actively transported to the nucleus via its dephosphorylation to facilitate CREB-dependent transcription of *Crh* (Jeanneteau *et al*, 2012). Thus, Jeanneteau and colleagues propose a mechanism in which GR signaling deactivates CREB function and *Crh* transcription by increasing the phosphorylation of CRTC2, which sequesters it in the cytoplasm (Jeanneteau *et al*, 2012). Supporting this possibility, GR-mediated repression of *Crh* is lost in rat primary hypothalamic neurons bearing a phosphor-deficient CRTC2 mutant. To influence CRTC2 phosphorylation and nuclear localization, GR signaling could influence the activity of the phosphatase calcineurin, since inhibition of calcineurin decreases *Crh* promoter activity to levels found following GR agonist treatment, but underlying mechanisms remain obscure.

Notably, other studies have suggested that stress-induced increases in circulating glucocorticoids may not be important for limiting *Crh* transcriptional responses. Accordingly, when challenged with a 3 hour restraint stressor, both ADX'd rats replaced with basal levels of CORT and sham-operated rats, exhibit increased CRH primary transcript levels after 30 minutes and a return to basal levels after 90 minutes (Shepard *et al*, 2005). These findings point to an important role for a glucocorticoid-independent player in regulating *Crh* expression. A role for inducible cAMP early repressor (ICER) has been identified (Aguilera and Liu, 2012; Shepard *et al*, 2005). ICER is an inhibitory member of the CREB family that can interact with pCREB to decrease cAMP-activated expression. A thorough review of the role of ICER in regulating *Crh* expression can be found in (Shepard *et al*, 2005). Nuclear corepressor (NCoR) and silencing mediator of the retinoid and thyroid hormone receptor (SMRT) may also be involved in such glucocorticoid-independent regulation of *Crh*, as one study revealed their importance for

repressing CREB-mediated induction of the *Crh* gene, but not for facilitating GR-mediated repression (Van Der Laan *et al*, 2008).

Regulation of *Crh* through the nGRE

The human *Crh* proximal promoter contains a functionally defined nGRE that mediates repression of *Crh* expression by glucocorticoids and can interact directly with GR (Malkoski *et al*, 1997; Malkoski and Dorin, 1999). This nGRE is composed of GRE and activator protein-1 (AP-1) half sites, which bind a GR monomer and a monomer of an AP-1 family member (i.e. c-Jun or c-Fos,), respectively; and the extent to which this nGRE directs repression depends on the AP-1 family member bound (Malkoski and Dorin, 1999). Given the proximity between the CRE and nGRE, it is possible that functional interactions exist between the GR and CREB at the level of the *Crh* proximal promoter (van der Laan *et al*, 2009). Moreover, as CREB binding protein (CBP) is a co-activator for pCREB as well as AP-1 proteins (Bannister *et al*, 1995; Bannister and Kouzarides, 1995), CBP might function as an important intermediary in such interactions. However, cAMP-stimulation of a simple reporter overexpressing CRE by was not affected by GR agonist treatment in cultured cells, arguing against an interaction of GR with the CREB signaling pathway (Van Der Laan *et al*, 2008).

A role for steroid receptor co-activator-1 (SRC-1)

Given the sequential and combinatorial assembly of protein complexes at the promoter by DNA bound steroid hormone receptors, it is likely that GR binding to a response element of the *Crh* gene is a pre-requisite for the recruitment of transcriptional co-regulators (Liu *et al*, 2001; Métivier *et al*, 2003). One player likely involved in such GR regulation of CRH via the nGRE is SRC-1. Although recruitment of SRC-1 to the *Crh* promoter by the nGRE bound GR has yet to be demonstrated, studies in cell lines have shown SRC-1 recruitment directly to the *Crh*

promoter via the estrogen receptor (Lalmansingh and Uht, 2008). Additionally, SRC-1 recruitment by the GR to a peculiar active GRE within the tree shrew *Crh* promoter, which increases *Crh* expression and susceptibility to stress, has been suggested (Fang *et al*, 2016). Thus, it is likely that GR similarly recruits SRC-1 when bound at the nGRE of *Crh*.

Although classically associated with transcriptional activation, both SRC-1 and steroid receptor co-activator-2 (SRC-2) have been shown to act as co-repressors of ligand-activated GR at certain GREs (Li *et al*, 2006; Rogatsky *et al*, 2002), and an essential role for SRC-1 in GR mediated repression of PVN *Crh* has been identified (Lachize *et al*, 2009). PVN *Crh* expression in SRC-1 knockout mice is resistant to down regulation by GR agonist treatment (Lachize *et al*, 2009). Moreover, a specific splice variant of SRC-1, SRC-1a, which is abundantly expressed in the PVN and contains a transcriptional repressor function, increases the sensitivity of the *Crh* promoter to GR-mediated repression in cultured cells (Meijer *et al*, 2000, 2006; Van Der Laan *et al*, 2008).

A role for DNA methylation

Another potential nGRE-centered mechanism of GR-mediated hypothalamic *Crh* repression involves methylation changes in the proximal *Crh* promoter and resulting recruitment of co-regulatory proteins. Treatment of a rat hypothalamic neuronal cell line with the GR agonist dexamethasone not only increases GR binding to the proximal *Crh* promoter, but also increases its interaction with a DNA methyltransferase (DnMT), DnMT3b, whose occupancy of *Crh* is also increased by dexamethasone (Sharma *et al*, 2013). Like other members of the DnMT family, DnMT3b facilitates methylation of CpG islands, such as those found in the proximal *Crh* promoter (Bestor and Verdine, 1994; McGill *et al*, 2006). Accordingly, both rat hypothalamic cells treated with dexamethasone (Sharma *et al*, 2013) and mice challenged with a social defeat

stressor (Elliott *et al*, 2010) exhibit CpG site specific increases in methylation within the proximal *Crh* promoter, although the pattern of methylation changes varies. Increases in DNA methylation can lead to transcriptional repression by interfering with transcription factor binding and/or by recruiting methylated DNA binding proteins (Comb and Goodman, 1990; Hendrich and Bird, 1998).

A number of MBD proteins have been identified (Methyl-CpG-binding proteins 1 and 2 (MeCP1 and MeCP2) and MBD1-4), and all but MBD3 specifically bind to methylated DNA *in vitro* and *in vivo* (Hendrich and Bird, 1998). Most of these MBD proteins could contribute to transcriptional repression of the *Crh* gene; however, MeCP2 is notable because loss-of-function mutations in MeCP2 cause Rett Syndrome, a severe neurodevelopmental disorder characterized by an abnormal stress response (Chahrour and Zoghbi, 2007). Unlike MeCP1, which has a high affinity for densely methylated DNA, MeCP2 can bind singly methylated CpG sites, such as those present in the *Crh* promoter (Elliott *et al*, 2010; McGill *et al*, 2006). Additionally, MeCP2 has been implicated in *Crh* transcriptional repression *in vivo* since increased PVN CRH mRNA is shown in mice expressing a mutated MeCP2 protein (McGill *et al*, 2006). Concurrently, GR agonist administration increases GR's interaction with MeCP2, as well as MeCP2's occupancy of the *Crh* promoter, in hypothalamic cells *in vitro*, where it presumably interacts with co-repressors to decrease transcription (Sharma *et al*, 2013). Together these studies point to significant involvement of MeCP2 in *Crh* regulation by GR, but this remains to be directly demonstrated.

MeCP2 has a well-established interaction with a histone de-acetylase (HDAC) and Sin3A containing co-repressor complex, which can impede the access of the transcription initiation complex to target promoters (Jones *et al*, 1998; Nan *et al*, 1998). HDAC1 specifically has been

implicated in the GR-mediated repression of *Crh*. In hypothalamic cells *in vitro*, GR agonist treatment increases HDAC1 interactions with GR and MeCP2, as well as HDAC1 occupancy of the *Crh* promoter (Miller *et al*, 2011; Sharma *et al*, 2013). Additionally, HDAC1, but not HDAC3, is important for GR-mediated repression of *Crh*, as only HDAC1 co-transfection restores dexamethasone-mediated repression of *Crh* promoter activity in hypothalamic cells treated with an HDAC inhibitor (Miller *et al*, 2011). Moreover, HDAC1 co-expression with CRH has been reported in the parvocellular region of the rat PVN, supporting its involvement in *Crh* regulation *in vivo* (Miller *et al*, 2011).

Collectively, these studies support a potential mechanism for GR-mediated *Crh* downregulation in which the ligand bound GR binds to the nGRE of the *Crh* promoter and recruits DnMT3b to methylate nearby CpG islands (Sharma *et al*, 2013) (Figure 1.5). This increased methylation could recruit MeCP2, which has well-established interactions with HDAC1. Notably, it is possible that such mechanisms do not involve GR binding to the nGRE at all. Rather, MeCP2 bound to methylated CpG sites could recruit the GR in order to stabilize MeCP2's interactions with HDAC1. The exact order of recruitment and assembly of these players requires further investigation, as does the necessity of DnMT3b and MeCP2 for GR-mediated repression of PVN *Crh*.

Histone modifications

Although histone acetylation is generally associated with activated gene expression, treatment of hypothalamic cells with a GR agonist has been reported to increase histone 4 acetylation, but not of histone 3, supporting a role for histone acetylation in repressing transcription (Miller *et al*, 2011). Additionally, GR regulation of *Crh* has been associated with increased trimethylation of histone 3-lysine 9, a marker of gene suppression, and unchanged

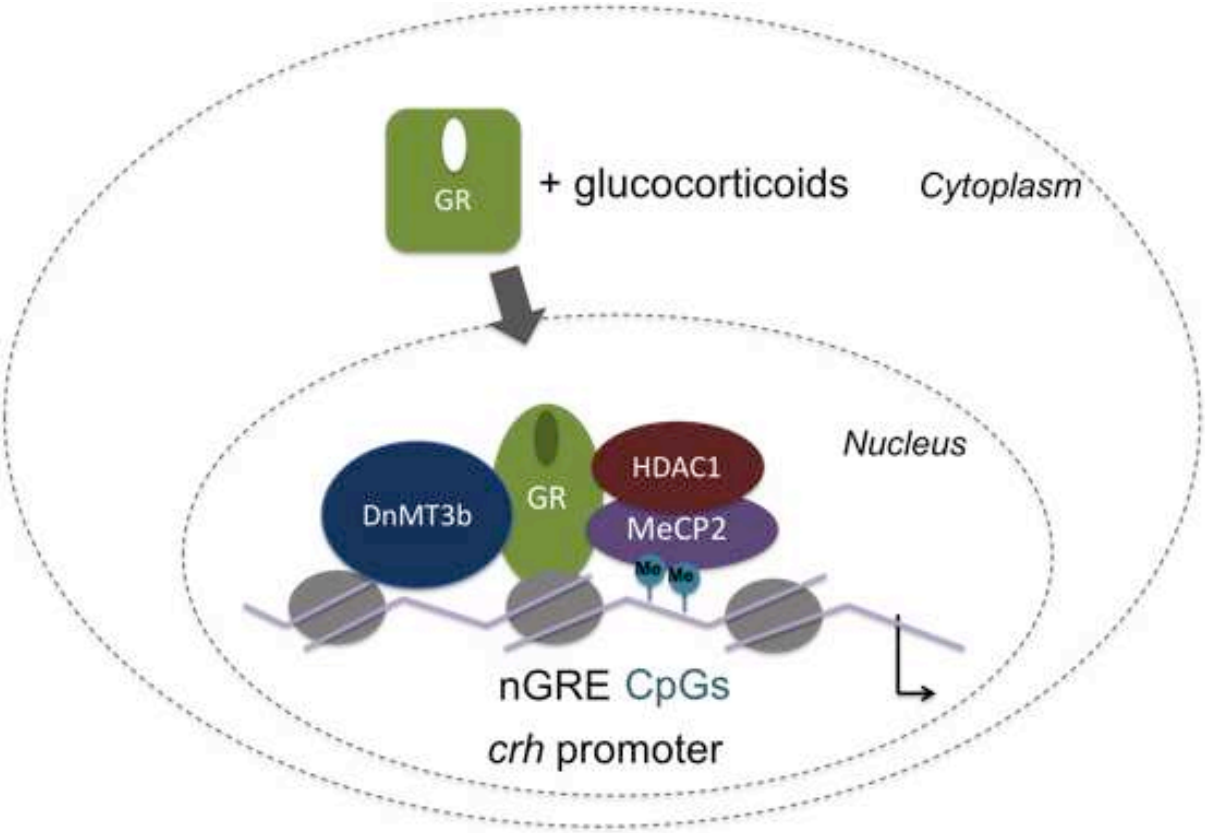

Figure 1.5. A putative glucocorticoid receptor (GR) repressor complex for the downregulation of corticotropin releasing hormone (*Crh*). In this potential mechanism, GR activated by high levels of glucocorticoids binds a negative glucocorticoid response element (nGRE) within the *Crh* promoter and recruits DNA methyltransferase 3b (DnMT3b) to methylate nearby CpG islands. This increased methylation potentially recruits Methyl-CpG-binding protein 2 (MeCP2), which interacts with histone de-acetylase 1 (HDAC1) to repress *Crh* transcription.

levels of trimethylated histone 3-lysine 4, an indicator of gene activation (Saccani and Natoli, 2002; Santos-Rosa *et al*, 2002; Sharma *et al*, 2013).

Conclusions

GR's repression of PVN *Crh* likely occurs through extremely complex interactions among proposed players at the CRE and/or nGRE, as well as other transcriptional regulators commonly associated with the GR but uninvestigated in the context of PVN *Crh* regulation. Such regulatory players likely influence changes in *Crh* gene expression via histone modifications that alter the accessibility of the transcriptional machinery to the *Crh* promoter, but this remains to be directly demonstrated. Moreover, it remains unclear how the transcriptional regulation of *Crh* by the GR meshes with evidence that corticosterone can regulate CRH translation and mRNA stability (Ma *et al*, 2001).

1.12 Addendum: Rhythms in hypothalamic pituitary adrenal (HPA) axis activity

Circadian rhythms are biological oscillations with a period of approximately 24 hours that exist throughout the body, including in the activity of the HPA axis and production of glucocorticoids (Spiga *et al*, 2014). Basal glucocorticoid levels fluctuate daily, with nadir levels found in the morning at the start of the light period (inactivity) and peak levels (5-10X nadir ones) found around the start of the evening dark period (active period) in rodents (Handa and Weiser, 2014). These rising levels are important for the onset of activity and feeding, which occurs during the evening in rodents and other nocturnal animals (Kalsbeek *et al*, 2012). Notably, the pattern of corticosteroid fluctuation is reversed in diurnal animals and humans, who are active during the day. As is the case for other circadian physiological functions, the circadian rhythm of the HPA axis is primarily controlled by a central clock in the suprachiasmatic nucleus (SCN) of the ventral hypothalamus, which is entrained by light input received from the retina

(Maywood *et al*, 2007; Spiga *et al*, 2014). To regulate the circadian rhythm of glucocorticoids, the SCN, in part, modulates the release of CRH from neurons in the PVN to alter the downstream activity of the HPA axis (Kalsbeek *et al*, 2012). The SCN has also been proposed to regulate adrenal activity via the autonomic nervous system. Specifically, a multisynaptic pathway from the SCN to the thoracic splachnic nerve is thought to facilitate autonomic regulation of the adrenal gland's sensitivity to ACTH (Buijs *et al*, 1999; Jasper and Engeland, 1997; Spiga *et al*, 2014). To add to the complexity, a circadian pacemaker is thought to exist within the adrenal and function independently of the SCN to regulate glucocorticoid production (Gi *et al*, 2008; Oster *et al*, 2006).

The basal activity of the HPA axis in rodents (Jasper and Engeland, 1991), like humans (Henley *et al*, 2009), also exhibits a rapid ultradian rhythm, characterized by a pulsatile pattern of CORT secretion with an inter-pulse interval of approximately one hour (Lightman *et al*, 2000). Although the ultradian rhythm of glucocorticoid secretion does not appear to depend on the SCN (Waite *et al*, 2012), it is thought to require appropriate levels of CRH secretion from hypothalamic neurons. In freely behaving rats, rapid fluctuations in CRH levels in the median eminence have been reported (Ixart *et al*, 1991, 1994), supporting the possibility that CRH drives pulses present in both ACTH and glucocorticoid secretion. However, other evidence suggests that CRH expression levels rather than pulses in its secretion are important for the ultradian pattern of glucocorticoid secretion (Walker *et al*, 2012). Alternatively, glucocorticoids can rapidly inhibit CRH-induced ACTH secretion, and such rapid glucocorticoid negative feedback on the HPA axis may also contribute to its oscillatory dynamics (Hinz and Hirschelmann, 2000; Spiga *et al*, 2014).

1.13 Addendum: The control of gonadal hormone production

Gonadal hormone production, which is essential for mediating reproductive functions, as well as diverse physiological functions, is tightly controlled by the hypothalamic-pituitary-gonadal (HPG) axis (Handa and Weiser, 2014) (Figure 1.6). The HPG axis is activated by gonadotropin releasing hormone (GnRH) synthesis in the basal forebrain and preoptic areas of the hypothalamus in the rodent, and in the medial basal hypothalamus of the human (Marques *et al*, 2000). Following its release from the hypothalamus into the hypophyseal portal vasculature of the median eminence, GnRH stimulates the synthesis of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by the anterior pituitary (Marques *et al*, 2000). These gonadotropins are released from gonadotrophs of the pituitary in response to GnRH pulses, which are typically around 30 minutes apart in the rodent. Specifically, high frequency and amplitude GnRH pulses preferentially stimulate the release of LH, whereas low frequency and amplitude pulses preferentially stimulate the release of FSH (Terasawa, 1998). LH and FSH act on the gonads (i.e. testes in males, and ovaries in females) to regulate steroid synthesis and gamete production (Marques *et al*, 2000). In females, FSH prompts the growth and maturation of ovarian follicles, and in combination with LH induces ovulation and estradiol synthesis by the ovary (Howles, 2000). In males, on the other hand, FSH acts with LH to promote spermatogenesis, while LH induces testosterone synthesis from the leydig cell of the testes (Kaprara and Huhtaniemi, 2018). As part of their diverse physiological activities, these gonadal hormones feed back on the hypothalamus and pituitary to inhibit the release of GnRH and LH and maintain the activity of the HPG axis within homeostatic limits (Maffucci and Gore, 2009). Importantly, estrogen-mediated negative feedback switches to a positive feedback mechanism

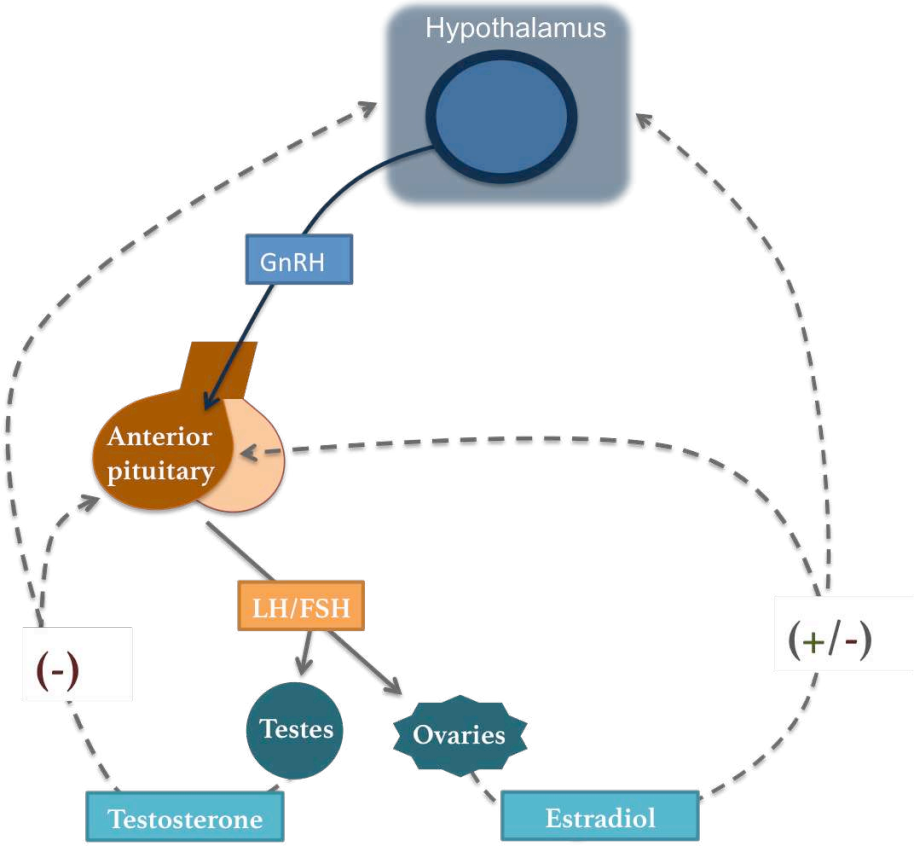

Figure 1.6. The hypothalamic pituitary gonadal (HPG) axis. Neurons in the hypothalamus secrete gonadotropin releasing hormone (GnRH) at the median eminence, which leads to the production of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the anterior pituitary. LH and FSH act on the testes in males, or ovaries in females, to drive the synthesis of testosterone and estradiol. Both testosterone and estradiol, in turn, feed back at the hypothalamic and anterior pituitary levels to inhibit HPG activity. Prior to ovulation in females, estrogen exerts positive rather than negative feedback.

just prior to ovulation in females in order to stimulate a pre-ovulatory GnRH/ LH surge that triggers ovulation in primates and rodents (Maffucci and Gore, 2009).

Once puberty has occurred in female rats and mice, adult animals experience an estrous cycle, across which a reoccurring sequence of events results in the maturation and release of follicles from the ovary approximately every 4-5 days. The estrous cycle is divided into four phases:, including diestrus I, diestrus II, proestrus, and estrus (Maffucci and Gore, 2009) (Figure 1.7). During diestrus I and II, GnRH and LH release are low, such that estradiol levels are at their lowest. The transition from diestrus II to proestrus is characterized by an increase in estrogen levels and a switch from negative estrogen-mediated negative feedback to positive feedback on the HPG axis. This induces the GnRH/LH surge, which triggers ovulation during estrus, as well as accompanying reproductive behaviors. For instance, females become more sexually receptive during the transition to estrus to increase the probability of successful fertilization (Gore, 2008; McCarthy, 2008). Plasma progesterone levels also peak in late proestrus and early estrus largely due to the presence of a corpus luteum, which is the transformed state of ovarian follicle post ovulation.

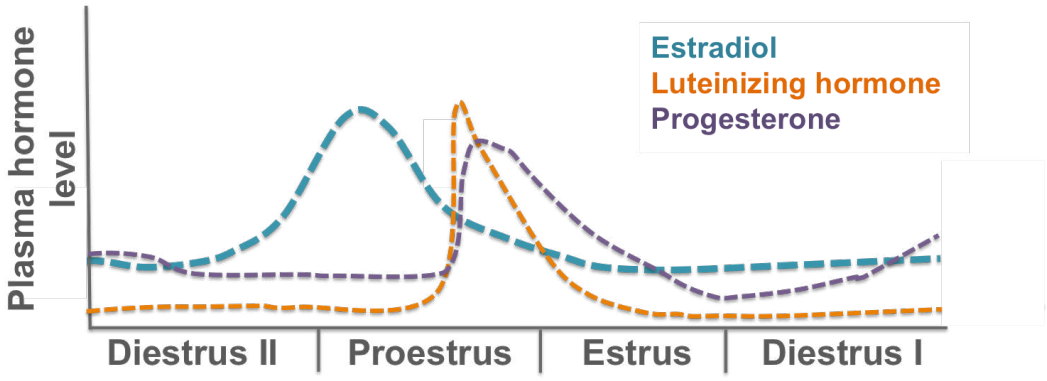

Figure 1.7. The estrous cycle of female rats and mice. Changes in plasma levels of estradiol (blue), luteinizing hormone (orange), and progesterone across this 4-5 day cycle are shown.

As is the case for all steroid hormones, the biosynthesis of gonadal hormones involves conversion from a common precursor, cholesterol, via the tissue-specific expression of necessary enzymes (Monroe and Spelsberg, 2004) (Figure 1.8). Testosterone and estradiol are the best-known forms of gonadal hormones belonging to the classes, androgens and estrogens, respectively. In addition to testosterone, androgens include, androstenedione, dihydrotestosterone, and dehydroepiandrosterone. Estrogens alternatively include, estradiol, estriol, and estrone. Although the gonads are the most prominent sources of these hormones, they can also be produced in the adrenal gland and locally within certain tissues, including brain, liver and adipose (Barakat *et al*, 2016). Gonadal hormone concentrations at a given tissue depend largely upon the expression levels of enzymes necessary for their biosynthesis.

1.14 Introduction of the present studies

Despite known sex differences in hypothalamic pituitary adrenal (HPA) axis activity and evidence for an underlying activational influence of gonadal hormones (reviewed above), few studies of glucocorticoid negative feedback on the HPA axis have examined both male and female subjects. Even fewer have studied sex differences in the glucocorticoid regulation of paraventricular nucleus (PVN) corticotropin releasing hormone (*Crh)* expression, an important nexus in both the activation and inhibition of the HPA axis. Impaired glucocorticoid negative feedback on hypothalamic CRH function may contribute to the HPA axis dysregulation commonly found in patients with stress-related psychological disorders (e.g. anxiety and depression), which exhibit sex-dependent prevalence (Bangasser and Valentino, 2014; Bao *et al*, 2008; Swaab *et al*, 2005). Thus, understanding how differing mechanisms mediate glucocorticoid control of PVN *Crh* expression in males versus females could lead to the identification of sex-dependent therapeutic approaches for the treatment of psychological

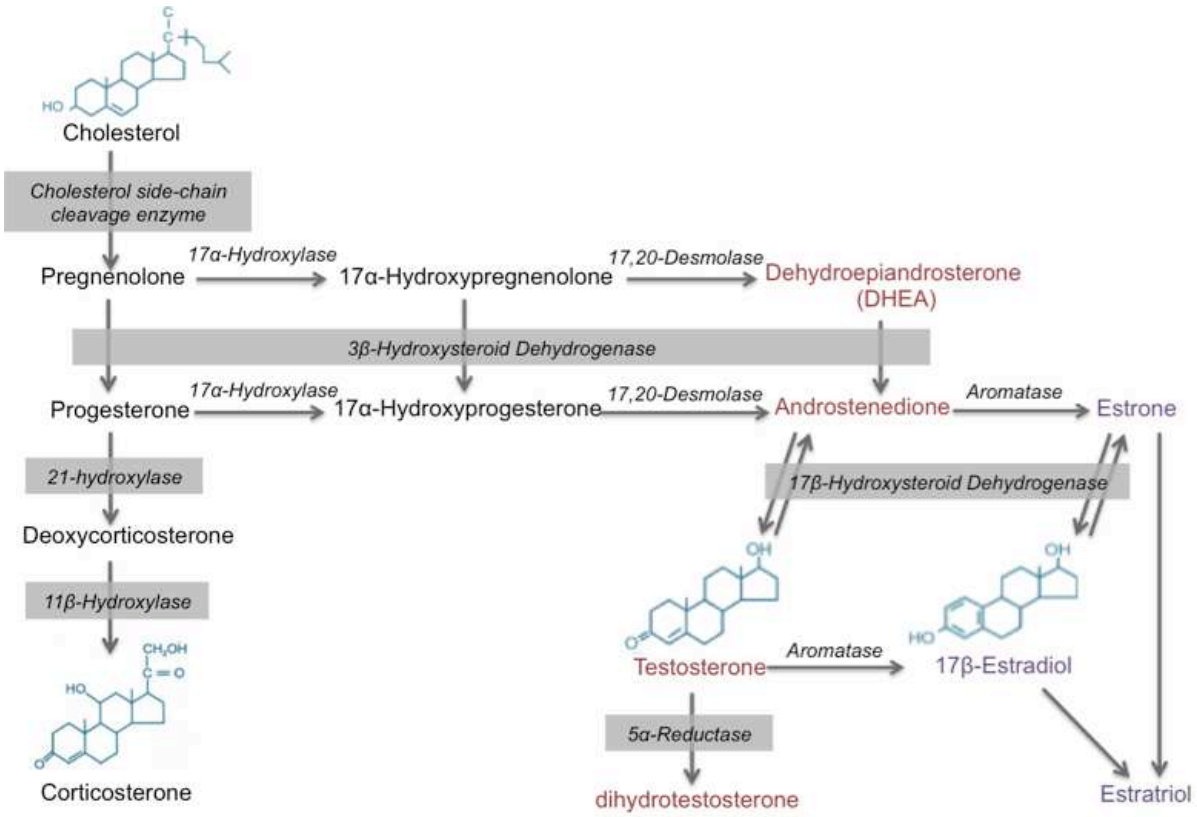

Figure 1.8. Steroid hormone biosynthesis. Steroid hormones, including estrogens, androgens, and glucocorticoids, are produced from a common precursor, cholesterol, via numerous enzymatic reactions. The enzymes responsible for each conversion strep are provided in italic font. Chemical structures illustrate differences between cholesterol and well-known examples of the glucocorticoid (i.e. corticosterone), androgen (i.e. testosterone), and estrogen (i.e. 17β-estradiol) classes. Members of the estrogen and androgen classes are indicated by purple and red text, respectively.

disorders. Accordingly, Chapters 2 and 3 of this dissertation addressed the overarching hypothesis that sex differences exist in glucocorticoid negative feedback on the HPA axis, which manifest at the level of PVN *Crh* due to an underlying influence of gonadal hormones.

In Chapter 2, the response of PVN *Crh* expression to the removal of glucocorticoid negative feedback by short-term adrenalectomy (ADX) in C57BL/6 mice was examined. A pronounced sex difference, in which males exhibited a more rapid increase in PVN *Crh* expression two days following ADX than did females, was detected. This sex difference depended on gonadal hormones, since the combination of ADX and gonadectomy eliminated the sex-related response of PVN *Crh* found following ADX alone. Moreover, this effect was dependent upon stimulatory actions of the potent androgen and testosterone metabolite, dihydrotestosterone (DHT), in males, which likely facilitated their more rapid *Crh* response to ADX than that of females. The limited co-expression of androgen receptors in PVN CRH neurons of male mice was also reported in Chapter 2 and points to DHT actions at limbic regions upstream of the PVN to control the expression of PVN *Crh* in the absence of glucocorticoid feedback (Bingaman *et al*, 1994a; Bingham *et al*, 2006; Williamson and Viau, 2007). Thus, the results of Chapter 2 support the hypothesis that gonadal hormone-dependent sex differences exist in the control of PVN *Crh* expression by glucocorticoids.

Chapter 3 more directly assessed sex differences in glucocorticoid negative feedback on PVN *Crh* by exploring sex-related mechanisms of glucocorticoid receptor (GR)-mediated transcriptional repression and the underlying influence of gonadal hormones in C57BL/6 mice. Following ADX, a greater decrease in PVN *Crh* expression in response to GR agonist treatment for two days was observed in female versus male mice, suggesting that females have more robust GR-mediated repression of *Crh* than do males. However, this was only the case if females were

examined on diestrus, the day of the estrous cycle when circulating levels of estradiol are lowest. If ADX'd females were studied on proestrus (high estradiol), the effect of GR agonist treatment to reduce PVN *Crh* expression was no longer observed. Sex- and estrous stage-dependent expression and/ or glucocorticoid regulation of proposed players in the GR transcriptional machinery were also seen, and these may underlie sex differences in GR-mediated negative feedback on PVN *Crh*. The results of Chapter 3 highlight a role for circulating estradiol to modulate the mechanisms of GR-mediated downregulation of *Crh*, ultimately providing further support for gonadal hormone-dependent sex differences in the glucocorticoid control of the HPA axis.

Together, the results of Chapters 2 and 3 led to a more complex question: Do sex differences in the glucocorticoid regulation of PVN *Crh* persist in times of chronic stress, a physiological model which is more etiologically relevant for human disease risk? A chronic variable stress (CVS) paradigm is used frequently in rodents to model the HPA dysregulation reported in some patients with stress-related diseases (Flak *et al*, 2011; Swaab *et al*, 2005); however the C57BL/6 mouse is typically found to be more resistant to CVS's effects (Chan *et al*, 2017). Thus, as C57BL/6 mice and transgenic mice on a C57BL/6 background were used for the studies in Chapters 2 and 3, it was necessary to first determine if CVS can in fact modulate the activity of the HPA axis in C57BL/6 mice. Accordingly, Chapters 4 and 5 tested the overall hypothesis that CVS alters the function of the HPA axis in C57BL/6 female and male mice. Specifically, effects of CVS on basic parameters of the HPA axis, including basal and acute stress-induced PVN neuropeptide gene expression and plasma corticosterone (CORT) levels in female (Chapter 4) and male (Chapter 5) mice were examined. Whereas female mice exhibited time-of-day dependent changes in the basal and acute stress-induced activity of the HPA axis

following CVS (Chapter 4), male mice initially appeared more resistant to its effects on the HPA axis, as previously suggested by Chan *et al*, 2017. Thus, studies in Chapter 5 determined that the presence or absence of social support, a factor that profoundly influences physiological and behavioral processes, including the HPA axis, alters the resilience of male C57BL/6 mice to CVS in the context of changing HPA axis function (DeVries *et al*, 2007; Kappel *et al*, 2017). These studies demonstrated that single- rather than pair-housed males predominantly exhibited altered PVN neuropeptide gene expression and stress-induced plasma CORT levels following CVS. Thus, although glucocorticoid negative feedback was not examined directly, these findings point to altered HPA function in female and single-housed male C57BL/6 mice. These results support the possibility that chronic stress alters glucocorticoid negative feedback and provide a foundation for future studies of sex differences in the glucocorticoid regulation of PVN *Crh* following chronic stress.

Collectively, the data in this dissertation highlight an important role for gonadal hormones as mediators of the sex-dependent glucocorticoid regulation of PVN *Crh*. At least in an acute scenario, the data support the possibility that a complex interplay among androgen/ estrogen actions and glucocorticoid regulatory mechanisms drives sex-biased *Crh* expression. Although sex differences in the glucocorticoid regulation of PVN *Crh* following chronic stress remain to be parsed out, the findings of this dissertation have established a foundation for future use of C57BL/6 mice for such studies. Continued investigations of this nature are essential, as they will help us to further understand how sex differences in the HPA response to acute stress may render one sex more vulnerable to stress-related disease following chronic stress.

Chapter 2: Androgens drive sex biases in hypothalamic corticotropin releasing hormone gene expression after adrenalectomy of mice[3]

2.1 Summary

Although prominent sex differences exist in the hypothalamic pituitary adrenal (HPA) axis's response to stressors, few studies of its regulation in the hypothalamic paraventricular nucleus (PVN) have compared both male and female subjects. In this study, we sought to explore sex differences in the acute regulation of PVN neuropeptide expression following glucocorticoid (GC) removal and the underlying role of gonadal hormones. We first examined the effects of short-term adrenalectomy (ADX) on PVN corticotropin releasing hormone (*Crh*) and arginine vasopressin (*Avp*) expression in mice using *in situ* hybridization. ADX increased PVN AVP mRNA levels in both sexes. In contrast, PVN CRH mRNA was increased by two days after ADX in males only. Both sexes showed increases in CRH mRNA after four days. To determine if gonadal hormones contributed to this sex bias, we examined adrenalectomized (ADX'd) and gonadectomized (GDX'd) mice with or without gonadal hormone replacement. Unlike the pattern in intact animals, two days following ADX/GDX, CRH mRNA levels did not increase in either sex. When males were given dihydrotestosterone propionate (DHTP), CRH mRNA levels increased in ADX'd/GDX'd males similar to those observed following ADX alone. To determine a potential mechanism, we examined the co-expression of androgen receptor (AR) immunoreactivity and CRH neurons. Abundant colocalization was found in the anteroventral bed nucleus of the stria terminalis, but not the PVN. Thus, our findings reveal a sex difference in

[3]Chapter 2 is modified from: Heck AL, Handa RJ, Androgens drive sex biases in hypothalamic corticotropin-releasing hormone gene expression after adrenalectomy of mice, Endocrinology, 2019, Vol. 160, No. 7, pg. 1757-1770, by permission of Oxford University Press

PVN *Crh* expression following the removal of GC negative feedback that may depend on indirect AR actions in males.

2.2 Introduction

All mammals, when faced with any environmental or homeostatic challenge, rely on activation of the hypothalamic pituitary adrenal (HPA) axis to adapt and survive. This key neuroendocrine system is activated by neurons in the hypothalamus in response to stressors and culminates in the synthesis of glucocorticoids (GCs; cortisol in humans or corticosterone (CORT) in rodents) by the adrenal cortex. Such stressor-induced elevations in GCs ultimately serve as first responders against threats to homeostasis. In such a situation, GCs mobilize energy stores to allow for appropriate physiological and behavioral responses (Munck *et al*, 1984; Sapolsky *et al*, 2000). However, the same GC response to stressors that is greatly beneficial in the short term can increase risk for stress-related diseases if persistently elevated (De Kloet *et al*, 2005; Holsboer, 2001; Sapolsky *et al*, 2000). Thus, understanding how GC production is regulated may have important therapeutic applications.

The HPA axis is principally controlled by neurons in the paraventricular nucleus (PVN) of the hypothalamus that release several neuropeptides during a stress response (Herman *et al*, 2003; Whitnall, 1993). These neuropeptides include corticotropin releasing hormone (CRH) and arginine vasopressin (AVP), which are co-expressed by some parvocellular PVN neurons and act together to stimulate the production of adrenocorticotropin (ACTH) by corticotrophs of the anterior pituitary gland (Antoni, 1993; Lolait *et al*, 2007; Muller *et al*, 2000; Rivier and Vale, 1983; Sawchenko *et al*, 1984; Schlosser *et al*, 1994; Vale *et al*, 1981). Increased ACTH in the systemic circulation acts on the adrenal cortex to induce the synthesis and release of GCs. GCs in turn down regulate their own production by inhibiting *Crh* and *Avp* gene expression in the PVN

(Aguilera and Liu, 2012; Ferrini *et al*, 1997). Therefore, PVN CRH and AVP co-expressing neurons represent an important nexus in the control of HPA activity by GCs.

Prominent sex differences exist in the activation of the HPA axis (Heck and Handa, 2019a). Following numerous stressors, neuronal activation is greater in the PVN of female versus male rodents (Larkin *et al*, 2010; Seale *et al*, 2004b; Viau *et al*, 2005). Females also have greater stress-induced levels of PVN AVP and CRH mRNAs (Babb *et al*, 2013; Iwasaki-Sekino *et al*, 2009; Viau *et al*, 2005). Such sex biases in the PVN have frequently been attributed to the opposing effects of gonadal hormones in adulthood (Handa and Weiser, 2014; Heck and Handa, 2019a). Accordingly, estrogens often are reported to increase, while androgens exclusively decrease, elevations in neuropeptide mRNAs brought on by varying types of stressors (Larkin *et al*, 2010; Lund *et al*, 2004b; Lunga and Herbert, 2004; Paulmyer-Lacroix *et al*, 1996; Seale *et al*, 2004b; Viau *et al*, 2003). Thus, gonadal hormones influence sex differences in the PVN, as well as those found at all levels of the HPA axis, to produce a more robust GC response to stress in female rodents (Handa and Weiser, 2014; Heck and Handa, 2019a).

Compared to the activation of the HPA axis, far less is known about sex differences in the inhibition of PVN neuropeptide expression by GCs. One study found that glucocorticoid receptor (GR) expression in the PVN is important for down regulating neuroendocrine stress responses in male but not female mice (Solomon *et al*, 2015). Yet, other studies of GC negative feedback that employ both male and female subjects are few and far between. Given that many stress-related diseases are associated with a dysregulation of GC responsiveness and exhibit a strong sex bias, it is essential that we understand HPA axis regulation in both sexes (Bangasser and Valentino, 2014). Thus, the present study aimed to explore sex differences in the control of PVN neuropeptide gene expression by GCs. We first examined the effects of short-term

depletion of circulating GCs by adrenalectomy (ADX) on PVN *Crh* and *Avp* expression in male versus female mice. We found that ADX more rapidly increased CRH mRNA in males. We next determined if gonadal hormones influenced this sex difference by using adrenalectomized (ADX'd) and gonadectomized (GDX'd) mice treated with or without gonadal hormones. Our findings reveal a sex difference unique to the timing of the *Crh* response to GC removal, which depends on androgen actions in males. To assess a potential mechanism for such androgen actions, we explored androgen receptor (AR) expression within CRH neurons in the PVN and the anteroventral BNST, an upstream HPA controlling region. By crossing B6(Cg)-Crh$^{tml(cre)Zjh}$/J (*Crh-IRES-Cre*) and B6.Cg-Gt(ROSA)26Sor$^{tml4(CAG-TdTomato)Hze}$/J (*Ai14*) strains, we generated *Crh-IRES-Cre;Ai14* mice in which CRH neurons are permanently tagged with a fluorescent protein. We identified abundant AR and CRH co-expression in neurons of the anteroventral BNST, but not the PVN, supporting a role for androgens to modulate indirectly the PVN *Crh* response to the removal of GC negative feedback.

2.3 Materials and Methods

Animals

Animals in these studies were housed in the laboratory animal research facility at Colorado State University. Subjects primarily included adult (2-4 month old) male and female C57BL/6N mice obtained from Charles River Laboratories (Wilmington, MA) or bred in house. If purchased, animals were allowed to acclimate for at least one week prior to use in experiments. If bred in house, mice were weaned at 21 days of age and group housed by sex. All animals were maintained in a 12 h light-12 h dark cycle with lights on at 6am, and provided *ad libitum* access to food and water.

Subjects included adult male *Crh-IRES-Cre;Ai14* mice. *Crh-IRES-Cre;Ai14* mice were generated from our colonies by crossing the B6(Cg)-Crh$^{tml(cre)Zjh}$/J (*Crh-IRES-Cre*) (Jackson Laboratories, Bar Harbor, ME; stock #012704, RRID:IMSR_JAX:012704) and B6.Cg-Gt(ROSA)26Sor$^{tml4(CAG-TdTomato)Hze}$/J (*Ai14*) (Jackson Laboratories; stock #007914, RRID:IMSR_JAX:007914) strains. These original stocks were generated as described previously (Madisen *et al*, 2010; Taniguchi *et al*, 2011). Genotyping was performed at weaning using an ear punch to identify *Crh-IRES-Cre* and *Ai14* mutants for use in the generation of heterozygous *Crh-IRES-Cre;Ai14* offspring as previously reported (Wamsteeker Cusulin *et al*, 2013). Adult male *Crh-IRES-Cre;Ai14* mice were used in subsequent experiments. These animals have previously been validated for the examination of CRH (Smith *et al*, 2014; Walker *et al*, 2018; Wamsteeker Cusulin *et al*, 2013) and the expression of CRH-immunoreactivity (ir) matches that of cre-driven reporter gene expression (Wamsteeker Cusulin *et al*, 2013). All animal surgeries and protocols were approved by the Institutional Animal Care and Use Committee at Colorado State University and were performed within NIH and AAALAC guidelines.

Experiment 1. Effect of time after ADX on PVN neuropeptide gene expression in male and female mice

Intact C57BL/6N male (n=5-8/group) and female (n=7-9/group) mice were bilaterally ADX'd under isoflurane anesthesia and given 0.9% saline as drinking water to maintain osmolarity. Sham operated males (n=5-7/group) and females (n=7/group) were given tap water. Two or four days following surgery, animals were decapitated within two minutes of first cage disturbance, and brains were removed, flash-frozen in 2-methylbutane at -40°C and stored at -80°C until sectioning for *in situ* hybridization to examine PVN gene expression. Tissue was also harvested for droplet digital PCR (ddPCR) from a separate cohort of sham (n=8) versus two-day

ADX'd (n=5) male mice. Upon decapitation, trunk blood was collected from all animals in chilled tubes containing 0.5 M ethylenediaminetetraacetic acid (EDTA) and aprotinin (4 mg/ml; Sigma-Aldrich, St. Louis, MO). Trunk blood was then centrifuged at 4°C, and plasma was removed and stored at -20°C until it was assayed for CORT by radioimmunoassay.

To control for the influence of the estrous cycle, all females in this experiment were killed on a day of diestrus when circulating estradiol levels are low. Cycle day was determined using vaginal cytology according to previously established methods (Goldman *et al*, 2007). Briefly, each female was lavaged daily with saline (0.9% NaCl) solution then samples were dried on glass slides and dipped in methylene blue (0.05%) for visualization using light microscopy. Estrous cycle stage was monitored daily for roughly two weeks prior to ADX to allow for habituation to handling and to enable prediction of estrous stage at time of death.

Experiment 2. Effect of GDX and ADX on PVN neuropeptide gene expression in both sexes

C57BL/6N male (n=4-6/group) and female (n=5-6/group) mice were bilaterally ADX'd and GDX'd under isoflurane anesthesia and kept on 0.9% saline or were sham operated and given tap drinking water. Two or four days following surgery, animals were killed, and brains were removed, flash-frozen and stored at -80°C until sectioning for microdissection and ddPCR. Upon decapitation, trunk blood was collected and centrifuged, and plasma was removed and stored as described in Experiment 1. Tissue harvested from sham and two-day ADX'd/ GDX'd male mice in this experiment was also examined alongside tissue from sham and two-day ADX'd male mice from Experiment 1 using ddPCR.

Experiment 3. Effect of androgen and estrogen treatment on PVN *Crh* expression in ADX'd and GDX'd males

C57BL/6N male mice (n=4-7/group) were bilaterally ADX'd and GDX'd as in Experiment 1. Starting at the time of surgery, males received daily subcutaneous (s.c.) injections of testosterone propionate (TP, Sigma-Aldrich, St. Louis, MO; 1mg/kg body weight (BW)), dihydrotestosterone propionate (DHTP, Steraloids, Newport, RI; 1mg/kg or 10mg/kg BW), 5α-androstane-3β, 17β-diol dipropionate (3β-diol P, Steraloids; 1mg/kg BW), estradiol benzoate (EB, Steraloids; 25 ug/kg or 250 ug/kg BW), or vehicle (27% hydroxypropyl-β-cyclodextrin, Cyclodextrin Technologies Development, Inc., Alachua, FL). Our objective with the delivery of DHTP was to roughly match total androgen levels of intact male mice, not just DHT levels, given that T can bind and activate the AR and can be 5α-reduced to DHT in tissue (Handa and Weiser, 2014). However, because of the broad range of androgen levels found in male mice (Lacombe *et al*, 2007; Okaji *et al*, 2012) the doses of DHTP were rough estimates of what might be seen *in vivo*. The lower doses for DHTP and EB have previously been shown to alter HPA activity in the male mouse (Lund *et al*, 2004a), and the higher doses were simply 10X the low dose amounts. The dose for TP has previously been shown to alter HPA axis activity in rats (Handa *et al*, 2013) and physiological function in mice (Blanqué *et al*, 2014); and the dose for 3β-diol P was determined using an allometric scaling calculator (http://clymer.altervista.org/minor/allometry.html) to adjust a dose (5 mg/kg) shown in our previous studies to alter *Avp* gene expression in the rat brain to one appropriate for the mouse (Pak *et al*, 2009). Two days following surgery, animals were decapitated and brains were collected and stored as above for ddPCR. Plasma was saved for CORT radioimmunoassay as described above.

CORT radioimmunoassay

Plasma levels of CORT were measured as previously described (Weiser and Handa, 2009) for all ADX'd animals in these studies to ensure the complete removal of the adrenal gland by ADX. Briefly, samples were diluted 1:25 in phosphate buffered saline (PBS) and then heated at 65 °C to denature corticosterone binding globulin, which can interfere with the assay. Diluted plasma samples (20 µl or 50 µl) were incubated overnight at 4°C with rabbit anti-CORT antiserum (1:1200, Cat# 7120016, RRID:AB_2801269, MP Biomedicals, Sonon OH) and ^{3}H-CORT (PerkinElmer, Boston, MA) in 0.01 M PBS containing 0.1% gelatin. Dextran coated charcoal was used to separate antibody bound CORT from free CORT. A standard curve ranging from 2.5 to 750 pg/tube CORT (Steraloids, Newport, RI) was run in every assay and used to quantify experimental samples. The inter- assay coefficient of variation, as measured by internal quality controls, was less than 10% for all assays. Animals were considered ADX'd if they had plasma CORT levels that were below the limit of detection (10 ng/ml). Only 3 animals had measureable levels of CORT and were removed from the study.

In situ hybridization

Brain sections 16-µm thick that contained the PVN were cut in the coronal plane using a CM3050 S cryostat (Leica, Wetzlar) into five series at -20°C, thaw mounted onto positively charged slides (Superfrost Plus, VWR Scientific, West Chester, PA), and stored dessicated at -80°C. For *in situ* hybridization, tissue was thawed to room temperature, fixed with 4% formaldehyde, acetylated with 0.25% acetic anhydride, delipidated in chloroform, dehydrated in a series of increasing ethanols and air-dried as previously described (Donner and Handa, 2009). 48-bp oligonucleotide probes for *Crh* (5'CAGTTTCCTGTTGCTGTGAGCTTGCTGAGCTAACTGCTCTGCCCGGGC-3') and *Avp*

(5'-GTAGACCCGGGGCTTGGCAGAATCCACGGACTCCCGTGTCCCAGCCAG=3') were end-labeled with [^{35}S] using terminal deoxynucleotidyl transferase (Thermo Scientific, Waltham, MA). Brain sections were then incubated at 37°C overnight in hybridization solution (50% formamide, .25% sodium dodecyl sulfate, 50 mM dithiothreitol .05% sodium thiosulfate, 600 mM NaCl, 10 mM Tris-HCl, 0.02% Denhardt solution, 1 mM EDTA, 0.01% denatured salmon testis DNA, 0.05% total yeast RNA, 0.005% yeast tRNA, 10% dextran sulfate) containing the radiolabeled probe at a concentration of 20×10^6 cpm/mL. After hybridization, sections were washed in 2X saline sodium citrate (SSC) with a final wash stringency of 1X SSC and then dehydrated in a series of ethanols ranging from 50-100%. To examine hybridization, slides were apposed to X-ray film (Carestream Kodak Biomax MR, Carestream, Rochester, NY) for up to four days (CRH) or 19 hours (AVP) to generate autoradiograms. Analysis of film autoradiograms was conducted using ImageJ software (version 1.51r). Optical density was quantified bilaterally throughout the rostral-caudal extent of the PVN in three (for CRH) or four (for AVP) adjacent and anatomically matched sections using a template of fixed size (1.8 μm^2). The density of exposed pixels was measured and expressed as arbitrary density units (AdU) for the PVN in all sections. Background activity in an adjacent area without labeling was subtracted from each measurement and resulting AdUs were averaged to obtain a single value per animal for statistical analysis.

Microdissection, RNA isolation and ddPCR

Frozen brains were sectioned at -16°C into 300-μm thick sections containing the PVN using a CM3050 S Cryostat (Leica, Wetzlar). PVN punches were obtained from two atlas matched thick sections per animal using a micropunch fashioned from sharpened heavy wall stainless steel type 304 tubing with an internal diameter of 0.991 +/- 0.0381 mm (Small Parts

Inc., Miami Lakes, FL). Tissue punches were kept frozen at all times and stored at –80°C until RNA extraction. Total RNA was isolated from PVN punches using the RNeasy mini kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Total RNA was reverse transcribed to yield cDNA using the iScript Reverse Transcription Super Mix (Bio-Rad, Hercules, CA). Droplet digital PCR was then used to measure target cDNA copies as a number of molecules. This approach shows increased sensitivity and decreased variability compared to other quantitative PCR methods (Hayden *et al*, 2013; Hindson *et al*, 2011; Miotke *et al*, 2014; Pinheiro *et al*, 2012). The ddPCR reaction mix was prepared by adding cDNA to an EvaGreen supermix (10µl; Bio-Rad, Hercules, CA) combined with forward (1µl) and reverse (0.4µl) primers and nuclease-free water up to a total volume of 20µl. Primers used are detailed in table 2.1, and all systems and reagents used for ddPCR were obtained from Bio-Rad (Hercules, CA). To generate droplets, the 20µl reaction mix and 70µl of droplet generation oil were added to specified wells in the DG8 Cartridge for the QX200 droplet generator, which was then inserted into the automated droplet generator. Post generation, droplets were transferred to a 96-well plate. The plate was sealed with foil using the PX1 PCR plate sealer and PCR amplification of template molecules in each individual droplet was accomplished in the C1000 Touch Thermal Cycler with 96-deep well reaction. The following thermal cycling protocol was used: 95°C for 10 min (1 cycle), 95°C for 30 sec then 60°C for 1 min (40 cycles), 4°C for 5 min (1 cycle), 90°C for 5 min (1 cycle), and hold at 4°C. The ramp rate was set at 2°C/sec, the sample volume at 40 μl, and the heated lid at 105°C. After PCR amplification, the plate was inserted into the QX200 Droplet Reader and the absolute template expression in copies/µl was quantified using QuantaSoft software. All values were normalized to the amount of cDNA loaded in each reaction, which was calculated based on cDNA concentrations quantified using the Quant-iT

OliGreen ssDNA Assay Kit (Thermo Scientific, Waltham, MA) according to manufacturer's instructions.

Table 2.1. Primers used for droplet digital PCR

Primer	Sequence (5' to 3')
CRH mRNA Forward	ATGCTGCTGGTGGCTCTGTC
CRH mRNA Reverse	GGATCAGAACCGGCTGAGGT
AVP mRNA Forward	TGCTCGCCAGGATGCTCAACAC
AVP mRNA Reverse	TTGCCGCCTCTTGGGCAGTT
GR mRNA Forward	GCAGTGGAAGGACAGCACAA
GR mRNA Reverse	GAGACTCCTGCAGTGGCTTG
MR mRNA Forward	GGCAAGCACTGCAACAGGTA
MR mRNA Reverse	GTCCTCTCTGCAGGTCCAAG
CRFR1 mRNA Forward	CTCTTCGCTCTGGGATGTCG
CRFR1 mRNA Reverse	CACTGCAGGCCAGAGACATT

Immunohistochemistry

Adult male *Crh-IRES-Cre;Ai14* mice (n=3) were intracardially perfused with ice-cold phosphate buffered saline (PBS; 0.01M; pH 7.4), followed by 4% paraformaldehyde (PFA) in phosphate buffer (PB, 4°C). Brains were removed from the skull and then placed in 4% buffered PFA for 24 hours and then infiltrated with 30% sucrose as a cryoprotectant. Four series of 35µm thick coronal sections were obtained using a Leica CM3050 S cryostat. Immunohistochemistry was performed on free-floating sections. Sections were washed 3 x 10 minutes in PBS and then quenched with 0.3% hydrogen peroxide in PBS, followed by 1 x 10 minute wash in PBS. Sections were incubated in blocking solution (10% normal goat serum (NGS) in PBS) for 1 hour at room temperature (RT) prior to incubation in primary antibody solution (10% NGS in PBS with 0.1% Triton-X 100 (PBST; pH 7.4)) overnight at RT. The primary antibody used was a rabbit anti-AR (1:500; catalog no. ab133273; RRID:AB_11156085; Abcam, Cambridge, MA), which has been previously validated (Chen *et al*, 2016; Swift-Gallant *et al*, 2016). Sections were then washed 3 x 10 minutes in tris-NaCl-tween (TNT; pH 7.4) buffer before incubation with a

biotinylated goat anti-rabbit secondary antibody (1:200; Vectastain Elite ABC Kit, Cat# PK-6101, RRID:AB_2336820, Vector Laboratories, Burlingame, CA) in TNT with 1.5% NGS for 1 hour at RT. Sections were washed 3 x 10 minutes in TNT and incubated for 30 minutes in TNT with 1:50 Reagent A plus 1:50 Reagent B from the Vectastain Elite ABC Kit according to manufacturer instructions (Vector Laboratories). Following 3 x 10 minute washes in TNT, sections were developed for 6 minutes using a Tyramide Signal Amplification Plus Fluorescein System (Perkin Elmer, Waltham, MA) per manufacturer instructions. 3 x 10 minute washes were performed in TNT, after which sections were mounted and coverslipped using ProLong Diamond Antifade Mountant (ThermoFisher Scientific, Waltham,MA).

Confocal imaging and colocalization quantification

Immunohistochemistry treated sections containing the rostral (Bregma -0.83 mm), middle (Bregma -0.95 mm), or caudal (Bregma -1.07 mm) PVN or the anteroventral BNST (Bregma 0.01-0.13 mm) (BNSTav) were identified using Paxinos and Franklin (2013) (Paxinos and Franklin, 2013). The BNST, lateral division, ventral part (STLV) and BNST, medial division, ventral part (STMV) were collectively defined as the anteroventral BNST (Gungor and Pare, 2016). Images were taken of each region bilaterally using laser scanning confocal microscopy. Z stacks composed of 0.83μm thick optical sections, spanning approximately 26μm were created for each image. Images for CRH:tdtomato neurons with AR-immunreactivity (-ir) were obtained using a Zeiss 880 laser scanning confocal microscope and a 20X (W Plan-Apochromat 20X/1.0 DIC Vis-ir ∞/0.17) objective.

Three-dimensional images were rendered from confocal Z-stacks with Imaris v9.1 software (Bitplane Inc, Zurich, Switzerland). CRH:tdTomato positive neurons and AR-ir positive neurons were automatically counted using Imaris v9 and manually checked.

CRH:tdtomato and AR-ir were considered to be colocalized when automatically determined cell centers were within 3μm of each other. Colocalization was confirmed visually, three dimensionally. Percentages of CRH:tdtomato neurons containing AR-ir were determined as the number of colocalized CRH:tdTomato neurons divided by the total number of these neurons X 100. For the PVN, numbers of total and colocalized CRH:tdtomato neurons were summed from bilateral counts at each imaged level (rostral, middle, or caudal). For the anteroventral BNST, numbers of total and colocalized CRH:tdtomato neurons were obtained from bilateral counts in one image.

Statistical methods

All data shown are mean values and all error bars represent SEM. In studies with only two treatment groups, unpaired Student's t-test was used for all pairwise data comparison. Statistical comparisons among three treatment groups were made using one-way ANOVAs. Fisher's Least Significant Difference post hoc test was used where appropriate. All statistics were done using the Prism statistical program (GraphPad Software, La Jolla, CA) and results were considered statistically significant when $p<0.05$. Additionally, all data were analyzed using the Extreme Studentized Deviate method (GraphPad) to detect significant outliers. Only three outliers were detected and excluded from further analyses.

2.4 Results

Experiment 1. Effect of time after ADX on PVN neuropeptide gene expression in male and female mice

To determine if GCs similarly regulate PVN neuropeptide gene expression in male and female mice, we examined the effect of ADX after two and four days on PVN CRH (Figure 2.1) and AVP (Figure 2.2) mRNA expression in both sexes using *in situ* hybridization. ADX

significantly increased CRH mRNA levels compared to those of sham-operated controls in both males ($F_{(2,13)}$=13.12; p<0.001) and females ($F_{(2,20)}$=29.31; p<0.0001) as determined by one-way ANOVAs. However, *post hoc* analyses revealed a sex difference in the effect of ADX based on time following surgery. Compared to intact controls, ADX'd males had significant increases in CRH mRNA levels after two (p<0.01) and four days (p<0.001). ADX'd females, alternatively, exhibited increased CRH mRNA only after four days (p<0.0001).

Whereas ADX increased PVN CRH mRNA in a sex-dependent fashion, it increased AVP mRNA levels in a similar pattern in both sexes (Figure 2.2). One-way ANOVAs revealed significant increases in AVP mRNA in both ADX'd males ($F_{(2,19)}$=7.3; p<0.01) and females ($F_{(2,19)}$=17; p<0.0001) compared to sham operated controls. Moreover, ADX'd males had significantly increased AVP mRNA after two (p<0.05) and four days (p<0.01) compared to intact controls. Likewise, ADX'd females showed increased AVP mRNA after two (p<0.0001) and four days (p<0.0001).

Experiment 2. Effects of gonadectomy (GDX) on ADX-induced PVN *Crh* and *Avp* expression

We next examined whether the sex-dependent effects of ADX on PVN *Crh* gene expression persist when gonadal hormone production is eliminated by GDX (Figure 2.3 a and b). When paired with GDX, ADX significantly increased CRH mRNA levels compared with control levels in males ($F_{(2,13)}$=5.396; p<0.05), and there was a strong trend in females ($F_{(2,15)}$=3.437; p=0.0591). *Post hoc* tests also revealed similarities between the sexes, as both males and females had significantly increased CRH mRNA expression four, but not two, days following surgery (males: p<0.01; females: p<0.05). Ultimately, GDX eliminated the sex difference in *Crh* gene expression at the two-day ADX time point that was observed in Experiment 1. To ensure that this was not a consequence of using ddPCR rather than *in situ* hybridization to measure mRNA

levels, we also demonstrated that, two days after ADX, males had significantly increased CRH ($p<0.05$; Figure 2.1e) and AVP ($p<0.01$; Figure 2.2e) mRNA when assessed by ddPCR.

In contrast to the response of CRH mRNA, GDX did not alter the effect of ADX on PVN AVP mRNA levels in males or females (Figure 2.3 c and d). The ADX and GDX combination significantly increased AVP mRNA levels compared with those of sham operated controls in both males ($F_{(2,12)}=7.578$; $p<0.01$) and females ($F_{(2,12)}=4.674$; $p<0.05$). Moreover, post hoc analysis showed increases in AVP mRNA at both two (males: $p<0.05$; females: $p<0.05$) and four days (males: $p<0.01$; females: $p<0.05$) after surgery, irrespective of sex.

To investigate how GDX alters the *Crh* response to ADX after two days, we examined changes in GC and CRH receptor gene expression following two-day ADX and ADX plus GDX. One-way ANOVAs revealed no effects of these procedures on GR or mineralocorticoid receptor (MR) mRNA levels (Figure 2.4 a and b). However, CRH receptor type 1 (*Crfr1)* expression was altered ($F_{(2,14)}=6.915$; $p<0.01$), and *post hoc* analyses showed significant decreases in CRFR1 mRNA levels in ADX'd and GDX'd males compared with both sham operated ($p<0.01$) and ADX'd ($p<0.05$) subjects (Figure 2.4c).

Experiment 3. Effects of androgen and estrogen treatment on PVN *Crh* expression in ADX'd and GDX'd males

To determine if androgens facilitate the increase in PVN *Crh* expression observed following two-day ADX, we examined the ability of TP and two doses of the non-aromatizable androgen DHTP to increase PVN CRH mRNA in ADX'd and GDX'd males (Figure 2.5a). One-way ANOVA revealed a significant effect of androgen treatment on CRH mRNA levels overall ($F_{(3,21)}=12.05$; $p<0.0001$). Specifically, TP treatment significantly decreased *Crh* gene expression

($p<0.05$), whereas the higher dose of DHTP significantly increased it ($p<0.01$), compared with that of vehicle-treated controls.

We also examined the ability of EB and 3β-diol P, an androgen metabolite that binds estrogen receptor β (ERβ) (Handa *et al*, 2009), to alter PVN *Crh* expression in ADX'd and GDX'd males (Figure 2.5b). Although one-way ANOVA showed a main effect of these treatments on CRH mRNA ($F_{(3,18)}=7.05$; $p<0.01$), *post hoc* analysis revealed a significant decrease in CRH mRNA only in 3β-diol P-treated animals compared with vehicle treated controls ($p<0.05$).

AR expression in CRH neurons

As DHTP treatment increased CRH mRNA, we next sought to determine if DHTP could act directly through ARs in PVN CRH neurons to increase *Crh* gene expression. We examined the expression of AR-ir in PVN CRH:tdtomato neurons (Figure 2.6). Tdtomato cells (4.69 +/- 0.78%) coexpressed AR-ir throughout the rostral-caudal extent of the PVN (Figure 2.6b). In the anteroventral BNST, however, 78.60 +/- 10.43% of tdtomato cells co-expressed AR-ir (Figure 2.6b).

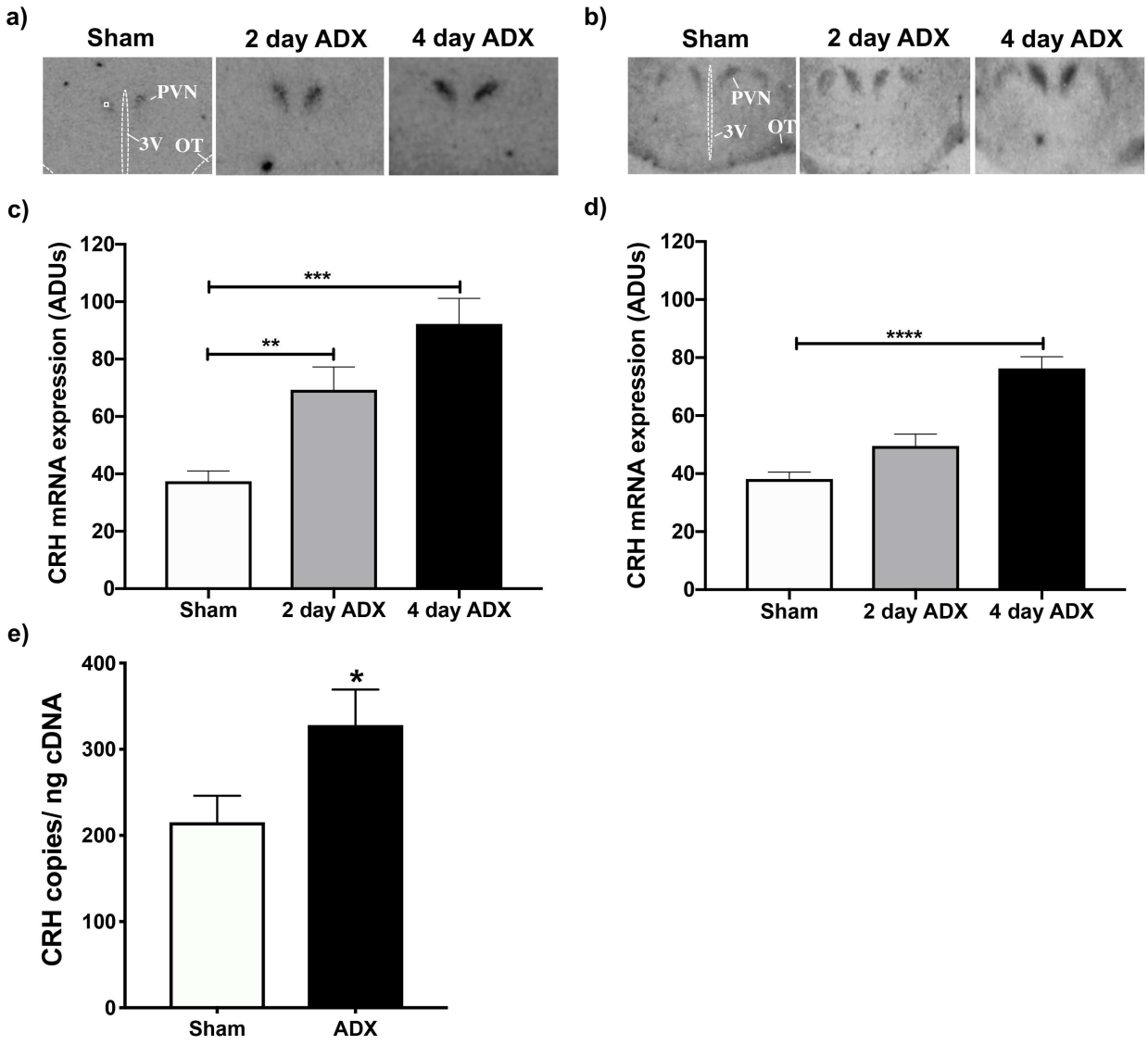

Figure 2.1. Changes in corticotropin releasing hormone (*Crh)* gene expression within the paraventricular nucleus (PVN) following adrenalectomy (ADX) of male and female mice. PVN CRH mRNA levels were evaluated using (a–d) *in situ* hybridization or (e) droplet digital PCR (ddPCR) in ADX'd male and female mice vs. sham-operated controls. (a and b) Representative autoradiograms of CRH mRNA hybridization in sham, 2-day ADX, or 4-day ADX (a) male or (b) female mice. (c and d) Arbitrary density units (AdUs) calculated from autoradiograms for (c) males and (d) females. Bars represent mean ± SEM AdUs in the PVN of n = 5 to 9 individuals. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. (e) Mean absolute levels ± SEM of PVN CRH mRNA in n = 5 to 8 male mice, 2 days after ADX or sham surgery. Absolute CRH mRNA levels were measured by ddPCR and normalized to the amount of input cDNA. $^{*}p < 0.05$ for ADX'd males compared with controls. 3V, third ventricle; OT, optic tract. □ in panel a depicts the size of the template used to calculate ADUs in Figures 1 and 2.

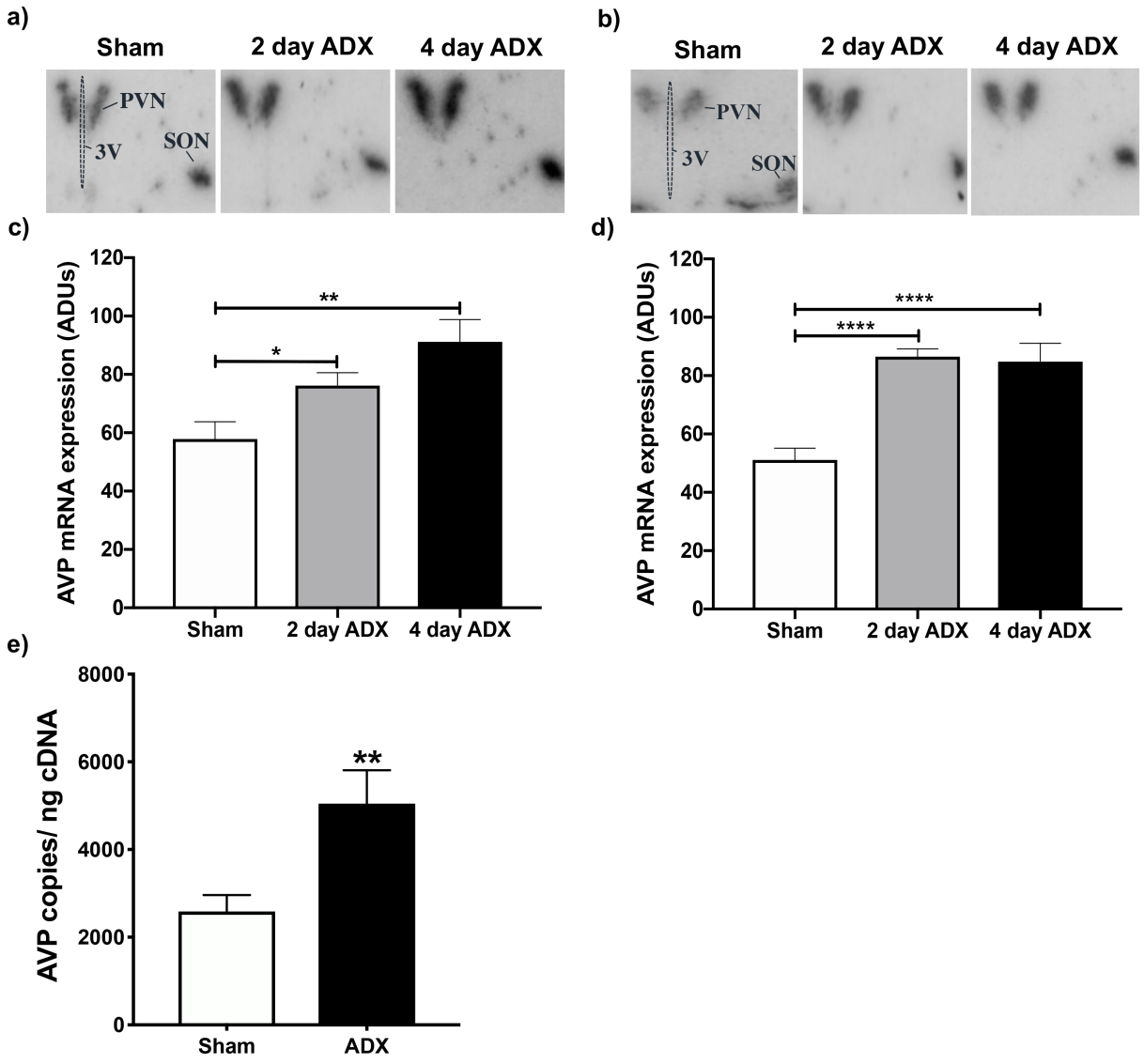

Figure 2.2. Changes in arginine vasopressin (*Avp*) gene expression within the paraventricular nucleus (PVN) following adrenalectomy (ADX) of male and female mice. PVN AVP mRNA levels were evaluated using (a–d) *in situ* hybridization or (e) droplet digital PCR (ddPCR) in ADX'd male and female mice vs. sham-operated controls. (a and b) Representative autoradiograms of AVP mRNA hybridization in sham, 2-day ADX, or 4-day ADX (a) male or (b) female mice. (c and d) Arbitrary density units (AdUs) calculated from autoradiograms for (c) males and (d) females. Bars represent the mean ± SEM AdUs in the PVN of n = 5 to 9 individuals. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$. (e) Mean absolute levels ± SEM of PVN AVP mRNA in n = 5 to 8 male mice, 2 days after ADX or sham surgery. Absolute AVP mRNA levels were measured by ddPCR and expressed relative to the amount of input cDNA. **$p < 0.01$ for ADX'd males compared with controls. 3V, third ventricle; SON, supraoptic nucleus.

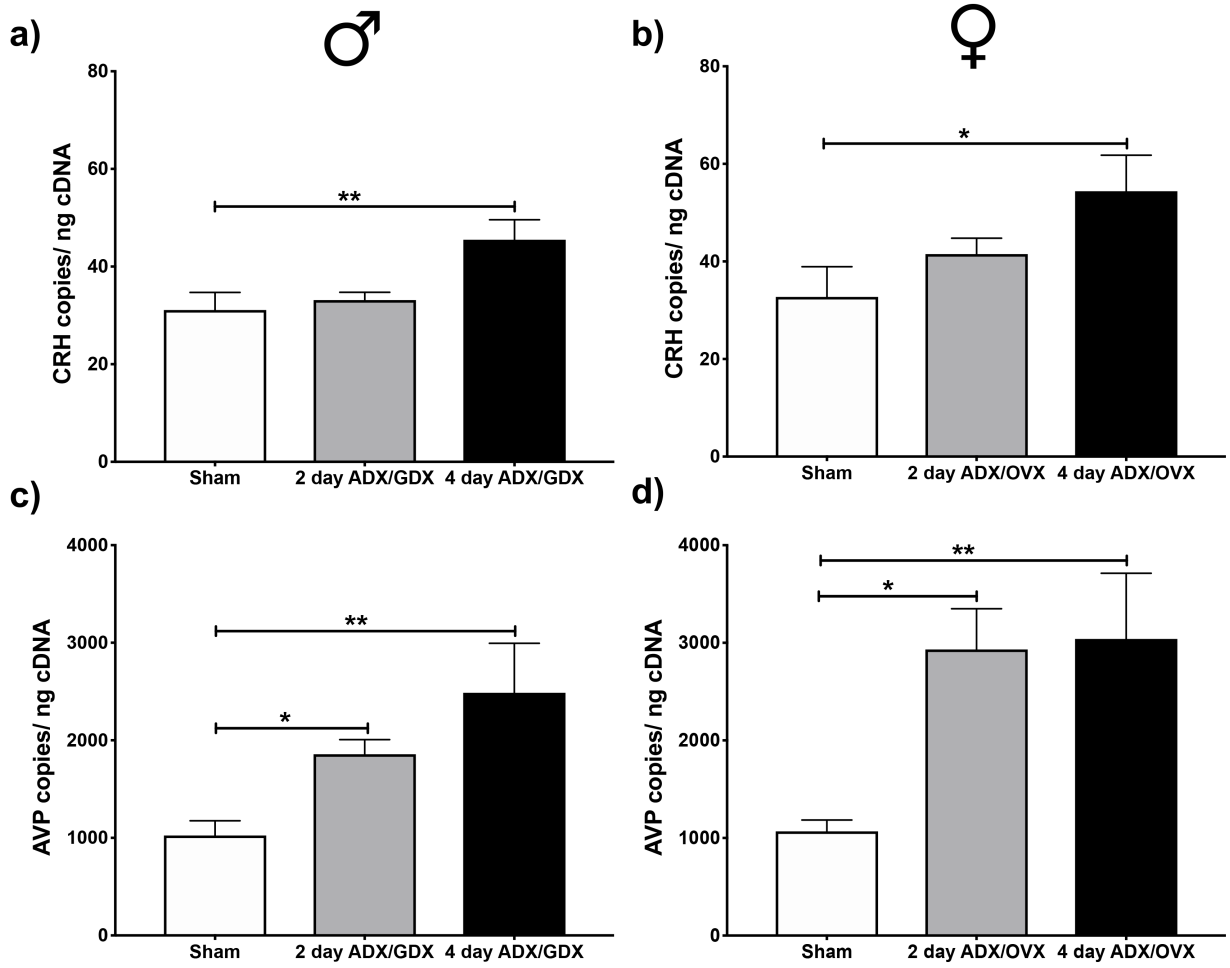

Figure 2.3. Changes in neuropeptide gene expression within the paraventricular nucleus (PVN) following adrenalectomy (ADX) and gonadectomy (GDX) of male and female mice. PVN CRH and AVP mRNA levels were evaluated in ADX'd and GDX'd male and female mice compared with sham-operated controls using droplet digital PCR (ddPCR). (a and b) Absolute levels of PVN CRH mRNA in sham, 2-day ADX and GDX, or 4-day ADX and GDX (a) male or (b) female mice. (c and d) AVP mRNA levels in (c) male or (d) female groups. All values were normalized to amount of input cDNA. Each bar represents mean normalized gene expression ± SEM of 4 to 6 mice. *$p < 0.05$; **$p < 0.01$. OVX, ovariectomized.

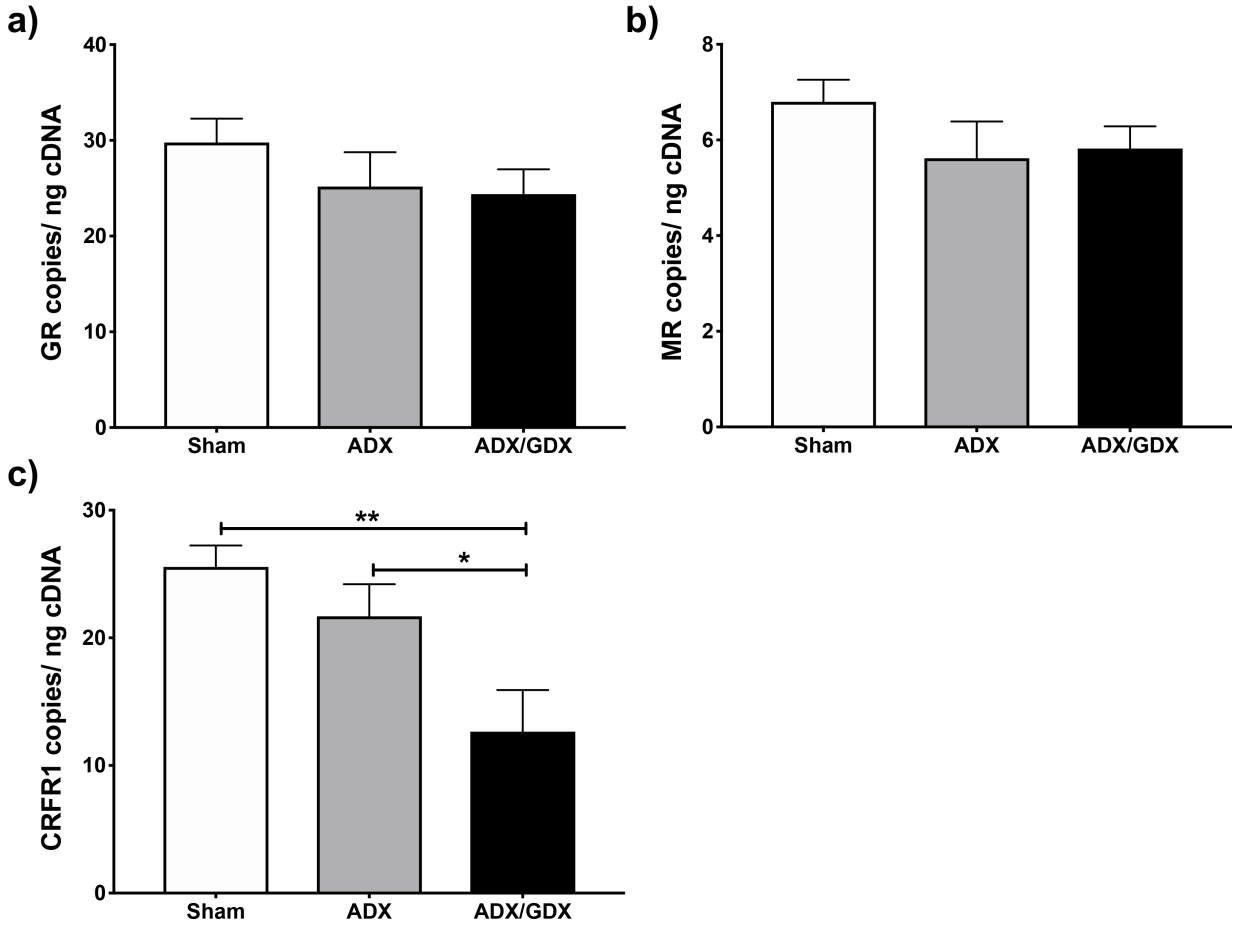

Figure 2.4. Changes in glucocorticoid and corticotropin releasing hormone (CRH) receptor gene expression within the paraventricular nucleus (PVN) 2 days following adrenalectomy (ADX) or ADX plus gonadectomy (GDX) of male mice. PVN glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and CRH receptor type 1 (CRFR1) mRNA levels were evaluated using droplet digital PCR (ddPCR) in ADX'd or ADX'd and GDX'd male mice and compared with those of sham-operated controls. Absolute levels of mRNA, normalized to the amount of input cDNA, are shown for PVN (a) GR, (b) MR, or (c) CRFR1. Each bar represents mean normalized gene expression ± SEM of n = 5 to 9 mice. *$p < 0.05$; **$p < 0.01$.

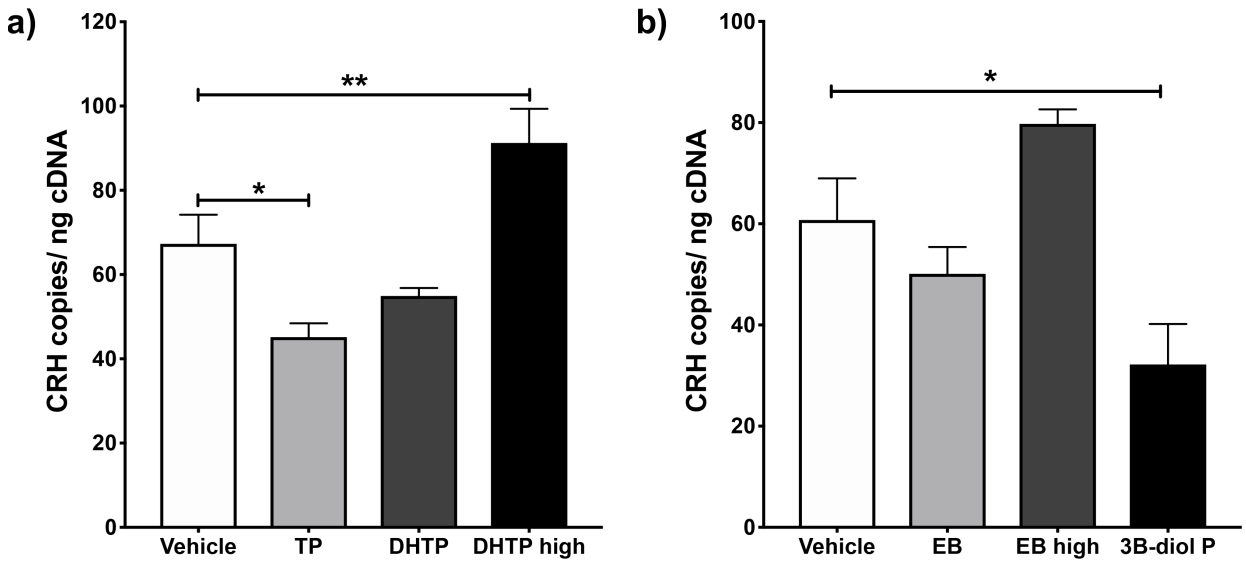

Figure 2.5. Effects of androgen or estrogen treatment on corticotropin releasing hormone (*Crh*) gene expression within the paraventricular nucleus (PVN) of adrenalectomized (ADX'd) and gonadectomized (GDX'd) males. (a) PVN CRH mRNA levels were measured in 2-day ADX'd and GDX'd male mice given testosterone propionate (TP; 1mg/kg bodyweight, s.c.), dihydrotestosterone propionate (DHTP; 1 or 10 mg/kg bodyweight, s.c.), or vehicle daily for 2 days. (b) PVN CRH mRNA levels were measured two days following ADX and GDX of male mice who were administered estradiol benzoate (EB; 25 or 250 ug/kg bodyweight, s.c.), 5α-androstane-3β, 17β-diol dipropionate (3β-diol P; 1 mg/kg bodyweight, s.c.), or vehicle daily for 2 days. Mean absolute levels of PVN CRH mRNA normalized to the amount of input cDNA ± SEM of n = 5 to 7 animals are shown. *$p < 0.05$; **$p < 0.01$.

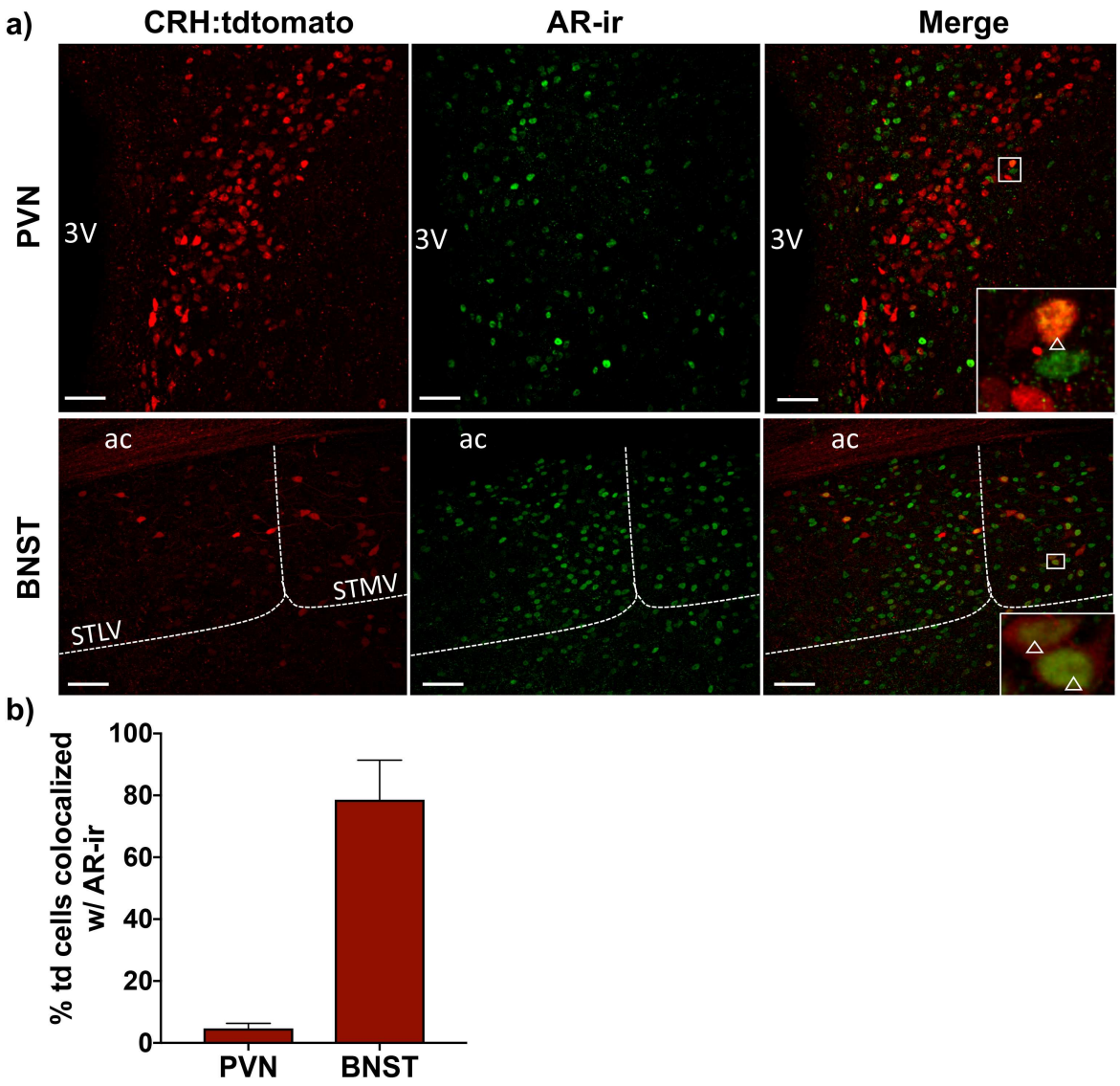

Figure 2.6. Androgen receptor (AR) expression in corticotropin releasing hormone (CRH) neurons. (a) Representative photomicrographs of brain sections taken from *Crh-IRES-Cre;Ai14* male mice containing the anterior PVN (Bregma –0.83 mm) and the anteroventral bed nucleus of the stria terminalis (BNST; Bregma 0.13 mm) and immunolabeled for AR are shown. (Left) CRH:tdtomato neurons in both brain regions; (middle) AR-immunoreactivity (ir); and (right) merged images at ×20 magnification. Magnified insets highlight coexpression of CRH:tdtomato and AR-ir that is present in the BNST but not the PVN. Open triangles indicate CRH:tdtomato neurons that express AR-ir. Scale bars, 50 µm. STMV and STLV are collectively defined as the anteroventral BNST, and dashed lines indicate the boundaries for each region. (b) Mean ± SEM percentages of CRH:tdTomato neurons (td) coexpressing AR-ir in the PVN and anteroventral BNST of 3 mice are shown. 3V, third ventricle; ac, anterior commissure; STLV, bed nucleus of the stria terminalis, lateral division, ventral part; STMV, bed nucleus of the stria terminalis, medial division, ventral part.

2.5 Discussion

In these studies, we identified a sex difference in the response of a key HPA axis regulatory gene to the removal of circulating adrenal GCs and explored the underlying influence of gonadal hormones. Our findings indicate that, whereas PVN *Avp* expression is upregulated following short-term ADX in both male and female mice regardless of gonadal hormone levels, PVN *Crh* expression is increased in a sex-dependent fashion. Two days after ADX, CRH mRNA was upregulated in males but not females, and GDX eliminated this sex difference, such that the male pattern was now more like that of the females. When given the potent, non-aromatizable androgen, DHTP, ADX'd and GDX'd males showed increased PVN CRH mRNA after two days as observed following the two-day ADX-alone protocol. Taken together, these findings suggest that sex differences in the acute effects of ADX on PVN *Crh* arise due to DHT actions in males. Such effects of DHT are likely mediated by indirect actions of ARs in the PVN or in upstream brain regions, as we observed limited AR expression in CRH:tdtomato neurons of the PVN.

Previous studies examining the effects of ADX on PVN neuropeptide gene expression have reported increases in both CRH and AVP hnRNA (Ma and Aguilera, 1999), mRNA (Ma and Aguilera, 1999; Makino *et al*, 1995b; Viau *et al*, 1999) and protein (Sawchenko, 1987b) levels. However, these studies have almost exclusively used male rats. The present studies fill a gap in our understanding of sex differences in the response to the removal of GC negative feedback by comparing both adult male and female ADX'd mice. In the short term, males responded more rapidly to the removal of GC negative feedback than females, as we demonstrated that CRH mRNA increased by two days after ADX in males but not until four days in females. Yet, both sexes exhibited increased AVP mRNA levels at both two and four days following ADX. Thus, females may initially rely more on GC regulation of PVN *Avp* versus *Crh*

to limit neuroendocrine stress responses. Supporting this, PVN AVP mRNA levels have been shown to be more sensitive to GC negative feedback than are levels of CRH mRNA (Makino *et al*, 1995b).

The more rapid increase in PVN CRH mRNA we observed in males versus females after ADX was eliminated if mice were GDX'd at the time of ADX. Thus, gonadal hormones are important for facilitation of the CRH mRNA response to ADX in male mice. In contrast, previous studies that used ADX'd and GDX'd male rats have shown that GDX inhibits *Avp*, but not *Crh*, transcriptional responses to ADX (Viau *et al*, 1999, 2001). A number of factors could contribute to this difference, including the species used or the duration of ADX and ADX/GDX. Viau et al. 1999 and 2001 examined ADX'd and ADX'd/GDX'd rats one to two weeks after surgery, whereas we studied more rapid changes occurring two or four days after surgery (Viau *et al*, 1999, 2001). Perhaps initially, PVN *Crh* is more sensitive to coregulation by gonadal hormones in the absence of GCs, and *Avp* eventually becomes more sensitive to this co-regulation. A more discrete time course of the effects of ADX versus ADX/GDX on PVN *Crh* and *Avp* gene expression is necessary to assess this.

Although GDX may prevent the acute PVN *Crh* response to two-day ADX in males via numerous mechanisms, one possibility is that it modifies the gene expression of GC and/or CRH receptors in the PVN to adjust the sensitivity of CRH neurons to regulation by GCs and CRH, respectively. We found no effect of short term ADX or ADX plus GDX on expression of the GC receptors MR and GR. Yet, the combination of ADX and GDX decreased expression of the primary receptor for CRH, *Crfr1*, relative to levels found in sham-operated controls and in ADX'd subjects. CRFR1 expression has been identified in PVN CRH neurons, especially following stress, where it likely activates *Crh* transcription via its coupling to adenylate cyclase

(Aguilera and Liu, 2012; Jezova *et al*, 1999; Luo *et al*, 1994; Makino *et al*, 1995a). Additionally, a distinct population of CRFR1-expressing neurons has been found in the mouse PVN that inhibits the activity of nearby hypophysiotrophic CRH neurons (Jiang *et al*, 2018; Justice *et al*, 2008; Ramot *et al*, 2017). Thus, it is possible that GDX decreases CRFR1 mRNA in PVN CRH neurons or in a nearby population to eliminate the increase in *Crh* transcription found following ADX, but further investigation of the molecular mechanisms underlying these possibilities is necessary.

Our findings suggest that the potent androgen, DHT, is important for facilitation of the acute (two-day) *Crh* response to ADX in males, as treatment with DHTP increased CRH mRNA in ADX'd and GDX'd males. Notable discrepancies exist between our observed stimulatory effect of DHTP on *Crh* and a body of literature suggesting that DHT suppresses ACTH and CORT secretion, partly by decreasing *Crh* gene expression in the PVN (Lund *et al*, 2004b, 2006; Seale *et al*, 2004b; Viau *et al*, 2003). Such discrepancies could reflect a dose dependency in the effects of DHT on components of the HPA axis, as a relatively lower dose of DHTP seemed to cause a slight, albeit nonsignificant ($p=0.1870$), decrease in CRH mRNA in our studies, matching the conclusions of previous studies (Lund *et al*, 2004b, 2006; Seale *et al*, 2004b; Viau *et al*, 2003). However, our findings related to treatment with a higher dose of DHTP do align well with studies demonstrating that DHT facilitates the *Avp* response to one week of ADX, as well as others that support a stimulatory or biphasic effect of DHT on the HPA axis (Viau *et al*, 2001). Stereotaxic placement of DHT in the BNST increases PVN *Avp* expression and stress-induced neuronal activation (Bingham *et al*, 2011b). Furthermore, GDX of male rats tends to decrease hypothalamic CRH content within the first week following surgery, whereas it significantly increases CRH content by week three (Bingaman *et al*, 1994b). Although the

potentially dose-dependent, excitatory effect of DHTP observed in the current studies is not unprecedented, the possibility that DHT has opposing effects, if administered at high and low doses, is certainly intriguing. There are several explanations for this apparent paradox that warrant further investigation, including the dose-dependent expression, activation and/or recruitment of ARs and/or AR-interacting transcriptional coregulatory proteins. DHT, at lower levels, could also be converted to and act through 3β-diol to decrease PVN CRH mRNA, an effect that might be overcome at higher androgen doses.

Interestingly, DHTP increased, whereas TP decreased, PVN CRH mRNA in ADX'd and GDX'd males. Because DHT is produced following testosterone (T) reduction by the 5α-reductase enzyme, and T and DHT both bind ARs with high affinity (even though DHT's is much greater than T's), T and DHT have often been reported to decrease similarly HPA activity (Handa *et al*, 1994b, 2013, Lund *et al*, 2004b, 2006; Viau and Meaney, 1996). Yet, T can also be converted to estradiol in brain tissue by the aromatase enzyme and may act through estradiol to influence PVN neuropeptide expression (Roselli *et al*, 1997). Our finding that neither a low nor a high dose of estradiol significantly altered PVN CRH mRNA in ADX'd and GDX'd males makes this scenario unlikely. Another possibility is that the differential expression and/or regulation of 5α-reductase in brain regions known to modulate HPA activity enable T to have distinct effects from DHT. Moreover, T, like DHT, could have dose-dependent effects on PVN CRH mRNA in these studies, but further investigation is ultimately necessary.

DHT can also influence the activity of the HPA axis through its metabolism in the brain and periphery to 5α-androstane-3β, 17β-diol (3β-diol), which binds selectively to ERβ (Lund *et al*, 2006). However, DHTP and its dipropionated metabolite, 3β-diol P, had opposing effects on PVN CRH mRNA in ADX'd and GDX'd males. Such opposing influences may be a result of

activation of different receptor types or activation of different receptor populations (Handa *et al*, 2009). 3β-diol can act directly on ERβs in the PVN to inhibit the HPA axis response to stress; whereas DHT likely acts on AR containing brain regions upstream of the PVN, as neurons directly controlling the HPA axis largely do not express ARs (Bingham *et al*, 2006; Handa *et al*, 1994a, 2009). Additionally, regulation of 3β-diol-synthesizing enzymes in the PVN by GCs and/or gonadal hormones may determine whether DHT or 3β-diol has a predominate influence on PVN *Crh* gene expression. Our data indicate that neither 3β-hydroxysteroid dehydrogenase type1 nor type 2 is expressed within neurons of the PVN regardless of GC or gonadal hormone status (data not shown). However, other enzymes (e.g., 3α-hydroxysteroid dehydrogenase) can act as surrogates for the production of 3β-diol and have been reported in the PVN (Handa and Weiser, 2014; Lund *et al*, 2006). Further investigation of 3β-diol synthesis in the PVN and in upstream brain regions will be important for the understanding of the opposing actions of DHT and 3β-diol in the acute regulation of the HPA axis.

In the present studies, we examined AR expression in CRH neurons by use of a transgenic mouse model (*Crh-IRES-Cre;Ai14*) in which CRH neurons stably express a fluorescent protein, tdtomato. This mouse model has been previously validated for the study of CRH neurons and enables accurate visualization of CRH without the need for manipulations typically used to enhance CRH-ir (Wamsteeker Cusulin *et al*, 2013). With the use of this model, we demonstrated that ARs are found in less than 5% of CRH:tdtomato neurons of the mouse PVN, suggesting that DHT may act predominantly on ARs outside of CRH neurons in the PVN or in upstream brain regions to exert its unique, permissive effects on the *Crh* transcriptional response to ADX. A previous study conducted in rats demonstrated an absence of AR in CRH neurons of the hypothalamus, septum, BNST and amygdala (Bingaman *et al*, 1994a). However,

this study likely did not examine entire populations of CRH neurons, as no method of enhancing CRH expression, such as colchicine treatment or ADX, was used. With the use of the *Crh-IRES-Cre;Ai14* mouse model, we have captured the entire population of CRH neurons to more accurately represent their coexpression of AR-ir. The coexpression of AR-ir in CRH neurons we found in the mouse PVN, although limited, parallels the findings of a previous study showing AR colocalization in some CRH neurons of the human PVN (Bao *et al*, 2006). Given that we do not know the percentage of colocalization in the human PVN, whether our findings indicate an interesting species-related difference in the androgen regulation of HPA function at the level of the PVN remains to be determined (Bao *et al*, 2006).

Although colocalization of CRH:tdtomato and AR-ir was limited in the PVN, we observed robust AR labeling within and outside of CRH:tdtomato neurons in the anteroventral BNST, a brain region well positioned to mediate the androgen regulation of the HPA axis. Anterior subdivisions of the BNST have neurons with AR expressing projections to the CRH containing parvocellular regions of the PVN (Williamson and Viau, 2007). Additionally, the anteroventral region of the BNST includes substantial populations of PVN projecting CRH neurons that are functionally distinct from GABAergic neurons in the region and have been suggested to participate in the activation of the HPA axis (Choi *et al*, 2007; Dong *et al*, 2001; Radley *et al*, 2009). DHT may enhance the activity of these BNST CRH neurons to increase *Crh* gene expression in the PVN in the absence of GCs. Our findings show that GDX selectively decreases PVN *Crfr1* expression when paired with ADX and support a role for CRH signaling in such androgen regulation of PVN *Crh*. Despite evidence for BNST to PVN CRH signaling, we cannot exclude the possibility that DHT inhibits the activity of GABAergic neurons in the anteroventral and other regions of the BNST that are not CRH expressing to increase PVN *Crh*

expression following ADX. However, results of one study argued against such a mechanism of GABA disinihition, as local DHT administration did not alter expression of GABA synthesizing enzymes in the posterior BNST, a region thought to play an especially prominent role in the inhibition of the HPA axis (Bingham *et al*, 2011b). Nonetheless, our findings certainly warrant further investigation of the role anteroventral BNST CRH-expressing and -nonexpressing neurons may play in mediating androgen regulation of the HPA axis, particularly following the release from negative feedback.

Ultimately, the results of these studies have shown a sex difference in the regulation of PVN *Crh* that is revealed in the absence of GC negative feedback and may depend on DHT actions outside of PVN CRH neurons in males. Our findings highlight the need for further systemic evaluation of central gonadal hormone effects on the HPA axis. Such studies will continue to advance our understanding of sex-biases in the prevalence of stress-related pathologies.

Chapter 3: Sex-dependent mechanisms of glucocorticoid regulation of the mouse hypothalamic corticotropin releasing hormone gene[4]

3.1 Summary

To limit excessive glucocorticoid secretion following hypothalamic pituitary adrenal (HPA) axis stimulation, circulating glucocorticoids inhibit corticotropin-releasing hormone (CRH) expression in paraventricular nucleus (PVN) neurons. As HPA function differs between sexes and depends on circulating estradiol (E2) levels in females, we investigated sex/estrous stage-dependent glucocorticoid regulation of PVN *Crh*. Using NanoString nCounter technology, we first demonstrated that adrenalectomized (ADX'd) diestrous female (low E2), but not male or proestrous female (high E2), mice exhibited a robust decrease in PVN CRH mRNA following two-day treatment with the glucocorticoid receptor (GR) agonist RU28362. Immunohistochemical analysis of PVN CRH neurons in *Crh-IRES-cre;Ai14* mice, where TdTomato fluorescence permanently tags CRH-expressing neurons, showed similarly abundant co-expression of GR-immunoreactivity in males, diestrous females, and proestrous females. However, we identified sex/estrous stage-related glucocorticoid regulation or expression of GR transcriptional co-regulators. Out of 17 co-regulator genes examined using nCounter multiplex analysis, mRNAs decreased by RU28362 in ADX'd mice in a sex/ estrous stage-dependent fashion included: GR (males = diestrous females > proestrous females), STAT3 (males < diestrous = proestrous), and HDAC1 (males < diestrous > proestrous). SRC-3, NCoR1, hnrnpu, CBP and CRTC2 mRNAs were lower in ADX'd diestrous and proestrous females versus males. Additionally, most PVN CRH neurons co-expressed MeCP2-immunoreactivity in diestrous

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female and male *Crh-IRES-Cre;Ai14* mice. Our findings collectively suggest that GR's sex-dependent regulation of PVN *Crh* may depend upon differences in the GR transcriptional machinery and an underlying influence of E2 levels in females.

3.2 Introduction

The neuropeptide corticotropin-releasing hormone (CRH) is a critical regulator of the body-wide adaptation to homeostatic challenges. In response to stressors, CRH synthesis dramatically increases in neuroendocrine neurons of the hypothalamic paraventricular nucleus (PVN) that direct the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Herman *et al*, 2003; Whitnall, 1993). The HPA axis orchestrates physiological and psychological reactions to stress by increasing production of adrenal glucocorticoids (GCs). Acute stress-related rises in GCs are beneficial, coordinating numerous physiological responses while negatively regulating PVN *Crh* to reduce their own production (Munck *et al*, 1984; Sapolsky *et al*, 2000). Yet, chronic, unrestrained rises in GCs increase risk for numerous stress-related disorders, partly due to disrupted negative feedback on the HPA axis (Dallman *et al*, 1992; De Kloet *et al*, 2005; Holsboer, 2001; Sapolsky *et al*, 2000).

To reduce *Crh* expression in PVN neurons and inhibit acute stress responses, GCs can act through either the mineralocorticoid receptor (MR) or GC receptor (GR) (Reul and de Kloet, 1985). However, the GR is thought to play a more predominant role in limiting PVN *Crh* expression in times of stress (Reul and de Kloet, 1985). The GR classically behaves as a nuclear receptor and, following ligand binding, translocates from the cytoplasm to the nucleus where it interacts with hormone response elements on DNA to directly decrease *Crh* transcription in the presence of high GC levels (Brink *et al*, 1992; de Kloet *et al*, 1998). The mechanism underlying the direct down regulation of PVN *Crh* transcription by the GR is not fully understood.

However, one possibility proposed by Sharma *et al*. is that GR activation recruits DNA methyl-transferase 3B (DnMT3b) to increase methylation of the *Crh* promoter and create binding sites for methylated CpG binding protein 2 (MeCP2) (Sharma *et al*, 2013). MeCP2 is thought to be a key player in the regulation of *Crh*, as CRH mRNA is increased in MeCP2 null mice (McGill *et al*, 2006) and MeCP2 interacts with the transcriptional repressor histone deacetylase 1 (HDAC1) (Sharma *et al*, 2013), which also participates in the GC repression of *Crh* (Miller *et al*, 2011). Other work has demonstrated an essential role for steroid receptor coactivator-1 (SRC-1) (Lachize *et al*, 2009). Moreover, GR signaling has been shown to limit *Crh* expression by opposing the excitatory influence of brain derived neurotrophic factor (BDNF), which involves tropomyosin receptor kinase B (TrkB)-cAMP response element binding protein (CREB) signaling (Jeanneteau *et al*, 2012). Ultimately, GR's repression of PVN *Crh* likely occurs through extremely complex interactions among these proposed players, and potentially other transcriptional regulators commonly associated with the GR but uninvestigated in the context of PVN *Crh* regulation.

Unfortunately, most studies examining the repression of PVN *Crh* by the GR have been performed *in vitro,* while *in vivo* studies have concentrated on male rodents. This is particularly problematic given known sex differences at all levels of HPA axis activation (Heck and Handa, 2019a), as well as emerging evidence for sex differences in GC negative feedback (Heck and Handa, 2019b; Solomon *et al*, 2015). In support of the latter, one study recently reported that GR expression by neurons of the PVN is necessary for negative feedback in male, but not female, mice (Solomon *et al*, 2015). Additionally, we recently demonstrated a more rapid response of PVN *Crh* expression to the removal of GC negative feedback by adrenalectomy (ADX) in male versus female mice (Heck and Handa, 2019b). As psychopathologies are often related to altered

GC negative feedback on the HPA axis and exhibit a striking sex difference in prevalence, continued investigations of sex differences in the GC regulation of PVN *Crh* are essential (Bangasser and Valentino, 2014).

In the present studies, we explored potential sex differences in the mechanisms of direct GC negative feedback on PVN *Crh* in adult mice. As fluctuations in circulating estradiol (E2) that occur across the female rodent estrous cycle can influence HPA axis activity (Viau and Meaney, 1991), we also examined females with low circulating E2 (diestrous) versus females with peak E2 levels (proestrous). We first demonstrated a pronounced sex difference in the involvement of the GR in the downregulation of PVN *Crh*. Adrenalectomized (ADX'd) diestrous female, but not male, mice exhibited a robust decrease in CRH mRNA following short term GR agonist treatment. However, proestrous ADX'd females did not have decreased CRH mRNA after the same treatment. Co-expression of GRs in PVN CRH neurons was similarly abundant in male, diestrous female and proestrous female *Crh-IRES-Cre;Ai14* mice, where the fluorescent TdTomato protein permanently tags CRH neurons. Thus, we sought to determine whether sex/ estrous stage-dependent differences in GR's repression of PVN *Crh* are accompanied by differences in the gene expression of the GR and/or its known transcriptional co-regulators, including DnMT3b, MeCP2, HDAC1, SRC-1 and players along the TrkB-CREB signaling cascade. We also examined other transcriptional regulators commonly associated with the GR but under investigated in the context of PVN *Crh* regulation. Taken together, our findings demonstrate sex differences in the GR transcriptional machinery that may depend on circulating E2 levels in females and help explain the presence of sex differences in GC negative feedback on *Crh* and HPA activity as a whole.

3.3 Materials and Methods

Animals

Animals in these studies were housed in the laboratory animal research facility at Colorado State University. They were maintained in a 12 h light-12 h dark cycle with lights on at 0600 h, and they were provided ad libitum access to food and water. All animal protocols were approved by the Institutional Animal Care and Use Committee at Colorado State University and were performed within NIH and AAALAC guidelines.

C57BL/6N mice: Adult (2-4 month old) male and female C57BL/6N mice were obtained from Charles River Laboratories (Wilmington, MA) and allowed to acclimate to the laboratory animal research facility for at least one week prior to use in experiments. At the start of experiments, intact C57BL/6N male (n=5-8/group) and female (n=5-7/group) mice were bilaterally adrenalectomized (ADX'd) under isoflurane anesthesia and given 0.9% saline as drinking water to maintain osmolarity. Beginning two days after surgery, ADX'd animals received one subcutaneous (s.c.) injection of the GR agonist RU28362 (0.4mg/kg) or vehicle (27% hydroxypropyl-β-cyclodextrin; Cyclodextrin Technologies Development, Inc., Alacua, FL) per day between 0900 h and 1000 h for two days. This dose of RU28362 is based on prior publications and was selected due to its ability to suppress HPA axis activity (Burgess and Handa, 1992; Francis *et al*, 2006). RU28362 is also highly selective for the GR, has little or no affinity for the MR (De Kloet, 1991; Reul and de Kloet, 1985), and like other synthetic glucocorticoids, likely has a relatively short half-life in plasma of around 2-4 hours, but longer lasting biological effects due to the long-lasting (12-36 hours) interaction of the ligand-receptor complex and ensuing transcriptional changes (de Kloet *et al*, 2017). Two days following the start of treatment, animals were injected with a third and final dose of RU28362 between 0900 and

1000 h and, 4 hours later, were anesthetized with isoflurane and decapitated in less than 2 minutes of the first cage disturbance. This occurred no later than 1400 h (lights on at 0600 h, off at 1800 h). Brains were removed, fresh frozen and stored at -80°C until they were sectioned for PVN microdissection and eventually droplet digital PCR (ddPCR) or N-Counter multiplexing to examine PVN gene expression.

Crh-IRES-Cre;Ai14 mice: Adult (2-4 month old) male and female *Crh-IRES-Cre;Ai14* mice were also used in these studies to enable identification of CRH expressing neurons without the use of immunohistochemical approaches, which are subject to limitations in peptide detection based upon GC-mediated downregulation of *Crh* expression. *Crh-IRES-Cre;Ai14* mice were generated from our colonies by crossing the B6(Cg)-Crh$^{tml(cre)Zjh}$/J (*Crh-IRES-Cre*) (Jackson Laboratories, Bar Harbor, ME; stock #012704, RRID:IMSR_JAX:012704) and B6.Cg-Gt(ROSA)26Sor$^{tml4(CAG-TdTomato)Hze}$/J (*Ai14*) (Jackson Laboratories; stock #007914, RRID:IMSR_JAX:007914) strains. These original stocks were generated as described previously (Madisen *et al*, 2010; Taniguchi *et al*, 2011). Genotyping was performed at weaning using an ear punch to identify *Crh-IRES-Cre* and *Ai14* mutants for use in the generation of heterozygous *Crh-IRES-Cre;Ai14* offspring as previously reported (Wamsteeker Cusulin *et al*, 2013). Adult male and female *Crh-IRES-Cre;Ai14* offspring were used to examine co-expression of GR and MeCP2 immunoreactivity (ir) in PVN CRH neurons. These animals have previously been validated for the examination of CRH (Smith *et al*, 2014; Walker *et al*, 2018; Wamsteeker Cusulin *et al*, 2013) and the expression of CRH-ir matches that of cre-driven reporter gene expression (Wamsteeker Cusulin *et al*, 2013).

Vaginal cytology

For studies comparing females to males, female C57BL/6N and *Crh-IRES-Cre;Ai14* mice were killed on a day of diestrus when circulating levels of E2 are low. A separate cohort of ADX'd vehicle- and RU28362-treated C57BL/6N females were also killed on proestrus to examine the influence of peak E2 levels on PVN gene expression. Cycle day was determined using vaginal cytology according to previously established methods (Goldman *et al*, 2007). Briefly, each female underwent vaginal lavage daily using a saline (0.9% NaCl) solution, then samples were dried on glass slides and dipped in methylene blue (0.05%) for visualization using light microscopy. For C57BL/6N female mice, estrous cycle stage was monitored daily for roughly two weeks prior to ADX to allow for habituation to handling and to enable prediction of estrous stage at time of death.

PVN microdissection and RNA isolation

Frozen brains were sectioned at -16°C into 300-μm thick sections containing the PVN using a CM3050 S Cryostat (Leica, Wetzlar, Germany). PVN punches were obtained from two atlas matched thick sections per animal using a micropunch fashioned from sharpened heavy wall stainless steel type 304 tubing with an internal diameter of 0.991 +/- 0.0381 mm (Small Parts Inc., Miami Lakes, FL). Tissue punches were kept frozen at all times and stored at −80°C until RNA extraction. Total RNA was isolated from PVN punches using the RNeasy mini kit (Qiagen, Germantown, MD) according to the manufacturer's instructions.

NanoString nCounter technology

RNA concentrations were measured using a Qubit 4 Fluorometer (Invitrogen, Carlsbad, CA). Multiplexed mRNA quantification was performed by the University of Arizona Genetics Core using a NanoString nCounter Custom CodeSet (Seattle, WA), which contained bar-coded

hybridization probes against mRNAs for CRH, arginine vasopressin (AVP), period circadian regulator 1 (Per1), GR, and GR co-regulators, including DnMT3b, HDAC1, MeCP2, SRC-1, steroid receptor coactivator 2 (SRC-2), steroid receptor coactivator 3 (SRC-3), nuclear corepressor 1 (NCoR1), CREB binding protein (CBP), death domain associated protein (DAXX), heterogeneous nuclear ribonucleoprotein U (hnrnpu), SIN3 transcription regulator family member A (Sin3a), signal transducer and activator of transcription 3 (STAT3), BDNF, CREB, TrkB, and CREB regulated transcription coactivator 2 (CRTC2). nSolver software was used for gene expression analysis, and, for each gene, transcript count was normalized to the geometric mean expression of four housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase, eukaryotic translation elongation factor 2, TATA-box binding protein, and beta-actin). Background expression level in each sample was calculated as the mean plus standard deviation of raw counts for eight synthetic negative control RNA probes for that sample. Only DnMT3b values were limited by this threshold. Specifically, two vehicle-treated males, two RU28362-treated males, three vehicle-treated diestrous females, four RU28362-treated diestrous females, two vehicle-treated proestrous females, and five RU28362-treated proestrous females had normalized DnMT3b transcript numbers at or below the background threshold. The threshold values in these cases were included only for calculation of approximate means but were not used for further statistical analyses.

Droplet digital (dd)PCR

Total RNA was quantitated using an Epoch Microplate Spectrophotometer and Gen5 v. 1.11 data analysis software (BioTek, Winooski, VT). cDNA was generated using an iScript cDNA synthesis kit (Bio-Rad, Munich, Germany). ddPCR was then used to measure target cDNA copies as a number of molecules. The ddPCR reaction mix was prepared by adding cDNA

to an EvaGreen supermix (10µl; Bio-Rad, Hercules, CA) combined with forward (1µl) and reverse (0.4µl) primers and nuclease-free water up to a total volume of 20µl. Primers used for CRH were: forward= 5'-ATGCTGCTGGTGGCTCTGTC-3' and reverse- 5'-GGATCAGAACCGGCTGAGGT-3'. All systems and reagents used for ddPCR were obtained from Bio-Rad (Hercules, CA). Droplet generation, PCR amplification of template molecules in each individual droplet, reading, and quantification of absolute template expression in copies/µl using QuantaSoft software were all performed as detailed previously (Heck and Handa, 2019b). All template expression values were normalized to the amount of cDNA loaded in each reaction, which was calculated based on cDNA concentrations quantified using the Quant-iT OliGreen ssDNA Assay Kit (Thermo Scientific, Waltham, MA) according to manufacturer's instructions.

Immunohistochemistry (IHC)

Adult male, diestrous female, and proestrous female *Crh-IRES-Cre;Ai14* mice (n=3-4) were intracardially perfused with ice-cold phosphate buffered saline (PBS; 0.01M; pH 7.4), followed by 4% paraformaldehyde (PFA) in phosphate buffer (PB, 4°C). Brains were removed from the skull, placed in 4% buffered PFA for 24 hours, and then infiltrated with 30% sucrose as a cryoprotectant. Four series of 35µm thick coronal sections were obtained using a Leica CM3050 S cryostat. IHC was performed on free-floating sections. 5 x 10 min washes were performed in PBS (0.01M; pH 7.4). Sections were then incubated in blocking solution (4% normal goat serum (NGS) in PBS) for 1 hour prior to incubation in primary antibody solution (PBS with 0.1% Triton-X 100 (PBST; pH 7.4) (plus 4% NGS for MeCP2 IHC)) overnight. Primary antibodies used were: previously validated rabbit anti-GR (Weiser *et al*, 2010) (1:500 dilution; Thermo Fisher Scientific, Waltham, MA, Cat# PA1-511A, RRID:AB_2236340) and rabbit anti-MeCP2 (1:1600; Cell Signaling Tech, Danvers, MA, Cat# 3456,

RRID:AB_2143849). Sections were then washed 3 x 10 minutes in PBST before secondary antibody incubation in the serum blocking solution. For MeCP2 IHC, Alexa Flour-488-conjugated goat anti- rabbit (1:1000; Thermo Fisher Scientific, Waltham, MA, Cat# A11008, RRID:AB_143165) was used as the secondary antibody and incubation occurred for 2 hours. Following 3 x 10 min washes in PBS, MeCP2 IHC treated sections were mounted and coverslipped using Vectashield H-1000 mounting medium for fluorescence (Vector Laboratories, Burlingame, CA). For GR IHC, biotinylated goat anti-rabbit (Vector Laboratories, Burlingame, CA, Cat# BA-1000, RRID:AB_2313606) was used as the secondary antibody and incubation occurred for 1 hour. Sections were washed 3 X 10 min in PBST then incubated for 1 hour in a tertiary antibody solution, containing Alexa Fluor-488-conjugated streptavidin (1:200; Thermo Fisher Scientific, Waltham, MA, Cat#S32354, RRID:AB_2315383) and 4% NGS in PBS. Following 6 x 10 min washes in PBS, sections were mounted and coverslipped using Vectashield. All wash and antibody incubation steps were performed on a shaker table at RT.

We also performed a preadsorption control for the anti-MeCP2 antibody in which the primary antibody was incubated with a 50X molar excess of immunizing peptide for three hours at RT prior to application to the tissue. The blocking peptide used was: human MeCP2 (Prospec, Rehovot, Israel). All other staining steps were completed as described above. Preadsorption of anti-MeCP2 resulted in the total loss of MeCP2-ir. Additionally, the widespread MeCP2 labeling throughout the brain in our studies matches the MeCP2 mRNA expression reported in the Allen in situ hybridization data set (https://mouse.brain-map.org/experiment/show/79904518) (Shen *et al*, 2012).

Confocal imaging and colocalization quantification

IHC-treated brain sections containing the PVN were identified using *Paxinos and Franklin's The Mouse Brain in Stereotaxic Coordinates* (Paxinos and Franklin, 2013), and images for CRH:TdTomato neurons with GR or MeCP2-ir were obtained using a Zeiss 880 laser scanning confocal microscope and a 20X (W Plan-Apochromat 20X/1.0 DIC Vis-ir ∞/0.17) objective. Confocal images were taken of the rostral (Bregma -0.83 mm), middle (Bregma -0.95 mm), or caudal (Bregma -1.07 mm) PVN bilaterally. Z stacks composed of 0.64μm thick optical sections, spanning approximately 29μm were created for each image.

Three-dimensional images were rendered from confocal Z-stacks with Imaris v9.1 software (Bitplane Inc, Zurich, Switzerland). CRH:TdTomato positive neurons and GR-ir or MeCP2-ir positive neurons were automatically counted using Imaris software (ver 9.1) and manually checked. CRH:TdTomato and GR-ir or MeCP2-ir were considered to be colocalized when automatically determined cell centers were within 4.5μm of each other. Colocalization was confirmed visually, in three dimensions. Percentages of CRH:TdTomato neurons containing GR-ir or MeCP2-ir were determined as the number of colocalized CRH:TdTomato neurons divided by the total number of these neurons times 100.

Statistical methods

All data shown are mean values, and all error bars represent SEM. For comparison of CRH:TdTomato and GR-ir or MeCP2-ir colocalization percentages, as well as CRH:TdTomato cells numbers, across the rostral to caudal extent of the PVN in male versus female mice, two-way repeated measures ANOVAs were performed. All other statistical comparisons were made using two-way ANOVAs. Fisher's Least Significant Difference *post hoc* test was used where appropriate. All statistics were done using the Prism statistical

program (version 8.0.1, GraphPad Software, La Jolla, CA) and results were considered statistically significant when $p<0.05$. Additionally, all data were analyzed using the Extreme Studentized Deviate method (GraphPad) to detect significant outliers. Only six outliers were detected and excluded from further analyses.

3.4 Results

Sex- and estrous stage-dependent effects of GR agonist treatment on PVN *Crh* expression

To determine if the GR similarly regulates PVN *Crh* expression in male and diestrous female mice, we examined the effect of treatment with the GR agonist RU28362 on PVN CRH mRNA levels in ADX'd mice of both sexes using ddPCR (Figure 1a). Two-way ANOVA showed significant main effects of RU28362 ($F(1,23)=12.27$; $p<0.01$), sex ($F(1,23)=6.87$; $p<0.05$) and a sex by RU28362 interaction ($F(1,23)=4.83$; $p<0.05$) on PVN CRH mRNA. *Post hoc* analyses revealed that RU28362 treatment did not significantly alter CRH mRNA levels in ADX'd males. However, it significantly decreased diestrous female CRH mRNA levels ($p<0.01$), largely due to significantly higher baseline CRH mRNA levels present in ADX'd females versus males ($p<0.01$).

Using nCounter technology, we not only validated our ddPCR findings, but we also examined females on proestrus, as well as diestrus, to determine whether estrous cycle stage influences GR-mediated negative feedback on PVN *Crh* (Figure 1b). Two-way ANOVA revealed a significant effect of sex/estrous stage ($F(2,29)=8.5$; $p<0.01$), but not of RU28362 treatment or interaction. Because we originally hypothesized that the *Crh* response to GR agonist treatment would depend on sex and estrous stage, we also performed *post hoc* analyses to examine the effects of RU28362 in ADX'd males versus diestrous females, as well as in proestrous females. A two-way ANOVA for males versus diestrous females exposed significant

effects of sex ($F(1,17)=6.27$; $p<0.05$) and RU28362 ($F(1,17)=8.31$; $p<0.05$); and *a priori* comparisons showed significantly higher CRH mRNA levels in vehicle-treated ADX'd diestrous females versus males ($p<0.05$), as well as significantly lower ($p<0.01$) CRH mRNA following RU28362 exclusively in diestrous females. In ADX'd proestrous females, alternatively, there was no significant decrease in PVN CRH mRNA following RU28362 treatment.

To determine if the absence of a significant effect of RU28362 in ADX'd male and ADX'd proestrous female mice was unique to PVN CRH mRNA, we examined changes in the expression of other GR-regulated genes. Two-way ANOVA revealed a significant effect of RU28362 ($F(1,31)=13.26$; $p<0.001$) to increase PVN Per1 mRNA in ADX'd mice, as well as a significant effect of sex/estrous stage ($F(2,31)=10.80$; $p<0.001$; Figure 1c). There was also a strong trend toward a significant RU28362 by sex/estrous stage interaction ($F(2,31)=3.232$; $p=0.0531$); therefore, we performed *post hoc* comparisons to examine the effects of RU28362 within male and female groups. RU28362 administration significantly increased PVN Per1 mRNA in ADX'd males ($p<0.001$, Figure 1c), but not females in either estrous stage. Furthermore, RU28362 decreased AVP mRNA levels in male and female ADX'd subjects (Figure 1d). Accordingly, a two-way ANOVA showed a significant effect of RU28362 ($F(1,31)=8.319$; $p<0.01$), but no significant effects of sex or interaction were found.

GR co-expression in PVN CRH neurons of both sexes

To determine whether differences in GR expression may differentially regulate CRH mRNA in male versus diestrous or proestrous female mice, we next examined the expression of GR-ir in PVN CRH neurons that were marked by the stable expression of a TdTomato fluorophore (CRH:TdTomato; Figure 2a). Two-way repeated measures ANOVAs (PVN level by sex/estrous stage) revealed significant effects of PVN level, but not of sex or interaction, on both

the percentage of CRH:TdTomato neurons colocalized with GR-ir ($F(1.132,7.921)=11.34$; $p<0.01$) (Figure 2b) as well as the number of CRH:TdTomato neurons ($F(1.172,8.202)=26.28$; $p<0.001$) (Figure 2c).

Sex- and estrous stage-dependent effects of GR agonist treatment on GR and co-regulator mRNAs

We next examined gene expression changes for the GR and its known co-regulators following vehicle or RU28362 treatment of male and diestrous or proestrous female ADX'd mice using nCounter technology (Figure 3). Two-way ANOVAs revealed significant effects of RU28362, to decrease GR mRNA ($F(1,31)=16.85$; $p<0.0001$; Figure 3a), as well as that of its co-repressors STAT3 ($F(1,31)=8.836$; $p<0.01$; Figure 3b) and HDAC1 ($F(1,30)=7.203$; $p<0.05$; Figure 3c). Although no significant effects of sex/estrous stage or interaction were detected for GR, HDAC1, or STAT3, we performed *post hoc* analyses based upon our original hypothesis that sex- and/or estrous stage dependent differences exist in the regulation of the GR transcriptional machinery by RU28362. RU28362 treatment significantly decreased GR mRNA in ADX'd males ($p<0.05$) and diestrous females ($p<0.01$), but not ADX'd proestrous females ($p=0.0893$). STAT3 mRNA levels, alternatively, were decreased following RU28362 treatment in ADX'd diestrous ($p<0.05$) and proestrous females ($p<0.05$), but not ADX'd males. HDAC1 mRNA levels were significantly decreased after RU28362 exclusively in ADX'd diestrous females ($p<0.05$). In contrast to the GR agonist-induced decreases in GR, STAT3, and HDAC1 mRNAs, RU28362 treatment significantly increased mRNA levels for the GR co-activator, SRC-1, regardless of sex or estrous stage (Figure 3d). Accordingly, a two-way ANOVA showed significant effects of RU28362 ($F(1,30)=14.63$; $p<0.001$) and of sex/estrous stage

($F(2,30)=4.364$; $p<0.05$), but not an interaction effect. Planned comparisons showed significant increases in SRC-1 mRNA levels following RU28362 in all groups ($p<0.05$).

Even though no effects of RU28362 treatment or RU28362 by sex/estrous stage interaction were observed for other GR co-regulatory genes examined, we found main effects of sex/estrous stage for SRC-3 ($F(2,30)=7.325$; $p<0.01$; Figure 4a), NCoR1 ($F(2,31)=6.806$; $p<0.01$; Figure 4b) and hnrnpu ($F(2,31)=6.670$; $p<0.01$; Figure 4c) by two-way ANOVAs. Specifically, SRC-3, NCoR1, and hnrnpu mRNA levels appeared lower in ADX'd females versus males. Table 1 shows other genes associated with GR co-regulation that were unaltered by sex/estrous stage, GR agonist treatment, or sex/estrous stage by RU28362 interaction.

Because GR can down-regulate PVN *Crh* expression by inhibiting *Crh* induction by BDNF (Jeanneteau *et al*, 2012), we also included genes associated with the BDNF regulation of *Crh* in the nCounter multiplex analysis study. No significant effects of RU28362 or RU28362 by sex/estrous stage interaction were identified for any of the genes examined (Table 1). However, we observed significant main effects of sex/estrous stage for CBP mRNA ($F(2,31)=3.993$; $p<0.05$; Figure 4d) and CRTC2 mRNA ($F(2,31)=4.458$; $p<0.05$; Figure 4e), where levels appeared lower in ADX'd female versus male mice.

MeCP2 co-expression in PVN CRH neurons of both sexes

We identified abundant co-expression of MeCP2-ir in CRH neurons throughout the rostral-caudal extent of the PVN of male (79.9% ± 5.5%) and diestrous female (84.8% ± 4.3%) mice that stably express the TdTomato fluorophore in CRH neurons (CRH:TdTomato) (Figure 5). No significant effects of sex, PVN level, or interaction were found by a two-way ANOVA with repeated measures across PVN level (Figure 5b).

Table 3.1. Mean +/- SEM normalized transcript numbers in the paraventricular nucleus (PVN) of ADX'd male, diestrous female and proestrous female mice measured using nCounter technology. $^{a(\#)}$ indicates that mean values represent the specified number of background threshold values.

	Males		**Diestrous Females**		**Proestrous Females**	
	ADX + Vehicle (n=5)	**ADX + RU28362 (n=6)**	**ADX + Vehicle (n=5)**	**ADX + RU28362 (n=7)**	**ADX + Vehicle (n=7)**	**ADX + RU28362 (n=7)**
GR repression						
DAXX	192.6 ± 3.9	195.8 ± 8.6	208.0 ± 7.8	198.9 ± 7.1	200.1 ± 5.5	198.8 ± 5.3
DnMT3b	38.4 ± 3.0$^{a(2)}$	33.6 ± 1.6$^{a(2)}$	33.9 ± 1.7$^{a(3)}$	32.5 ± 1.6$^{a(4)}$	33.4 ± 1.5$^{a(2)}$	31.4 ± 1.2$^{a(5)}$
MeCP2	1172.7 ± 39.4	1244.3 ± 29.2	1130.1 ± 14.3	1208.2 ± 65.7	1114.0 ± 15.7	1197.9 ± 81.8
Sin3a	1131.0 ± 41.4	1119.7 ± 76.6	1145.0 ± 39.4	1112.8 ± 24.0	1161.1 ± 46.6	1046.8 ± 45.1
SRC-2	3217.8 ± 142.8	3689.9 ± 108.8	3530.3 ± 91.6	3497.9 ± 139.0	916.9 ± 33.7	928.0 ± 46.0
BDNF activation						
BDNF	125.5 ± 2.9	133.4 ± 10.0	119.1 ± 8.2	124.5 ± 9.8	123.2 ± 11.6	115.1 ± 6.3
CREB	987.7 ± 31.7	967.8 ± 21.5	930.1 ± 30.9	950.7 ± 15.7	923.4 ± 21.6	939.9 ± 31.8
TrkB	12938.9 ± 246.2	13389.8 ± 652.7	13090.2 ± 387.9	13131.0 ± 293.7	11734.9 ± 344.8	12896.7 ± 254.8

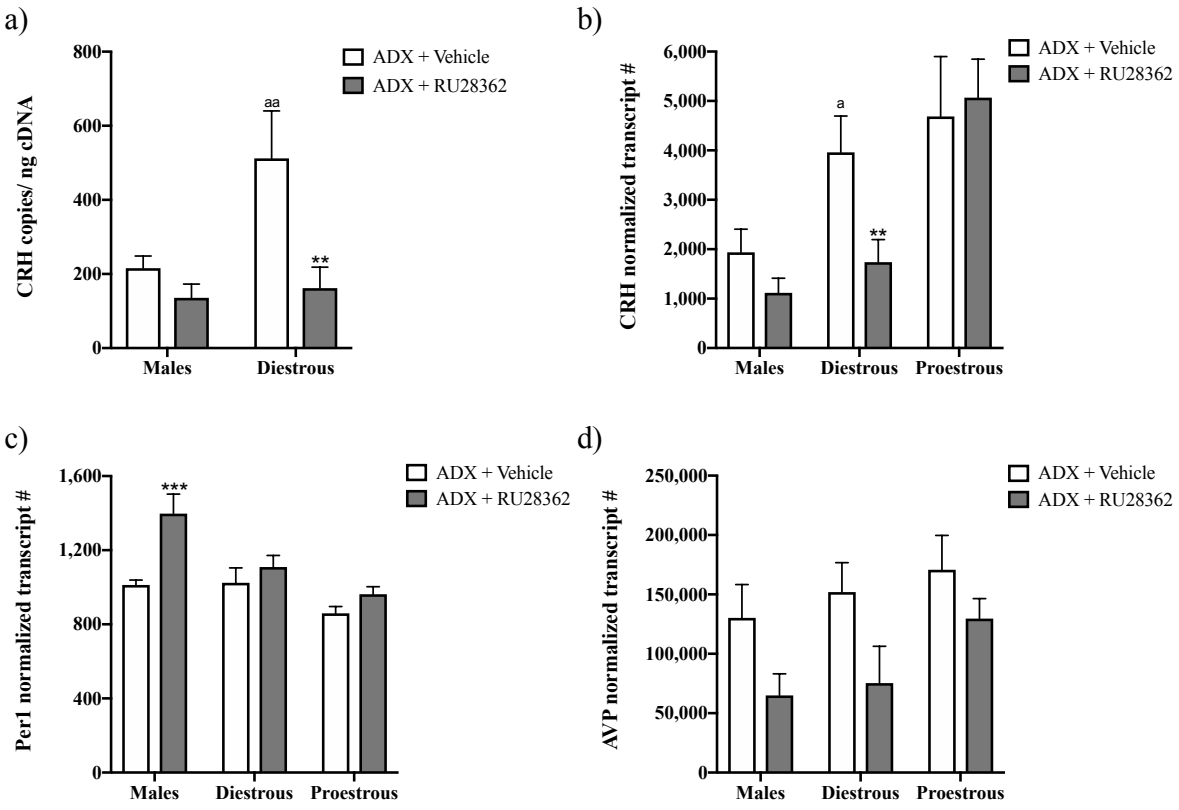

Figure 3.1. Effect of glucocorticoid receptor (GR) agonist treatment on expression of glucocorticoid-regulated genes within the hypothalamic paraventricular nucleus (PVN). Changes in PVN gene expression in adrenalectomized (ADX'd) male, diestrous female, or proestrous female mice treated with RU28362 (0.4mg/kg s.c.) or vehicle are shown. Panels a and b show CRH mRNA levels in males and females measured using droplet digital (dd)PCR (a) or nCounter technology (b). Panel c shows period circadian regulator 1 (Per1) mRNA levels, and panel d shows arginine vasopressin (AVP) mRNA levels measured using nCounter technology. For the nCounter technology, mRNA levels were normalized to the geometric mean expression of four housekeeping genes. For ddPCR, mRNA levels were normalized to the amount of input cDNA. Each bar represents the mean normalized gene expression +/- SEM of n=4-8 mice. Two-way ANOVAs revealed main effects of RU28362 for CRH ((a); $p<0.01$), Per1 ($p<0.001$), and AVP ($p<0.01$) mRNAs. Main effects of sex/estrous stage were found for CRH ((a): $p<0.05$; (b): $p<0.01$) and Per1 ($p<0.001$), and interaction effects were found for CRH ((a);$p<0.05$) and Per 1 ($p=0.0531$). a=$p<0.05$ and aa=$p<0.01$ for diestrous females versus males of the same experimental group. **=$p<0.01$ for RU28362- versus vehicle-treated diestrous female mice. ***=$p<0.001$ for RU28362- versus vehicle-treated male mice.

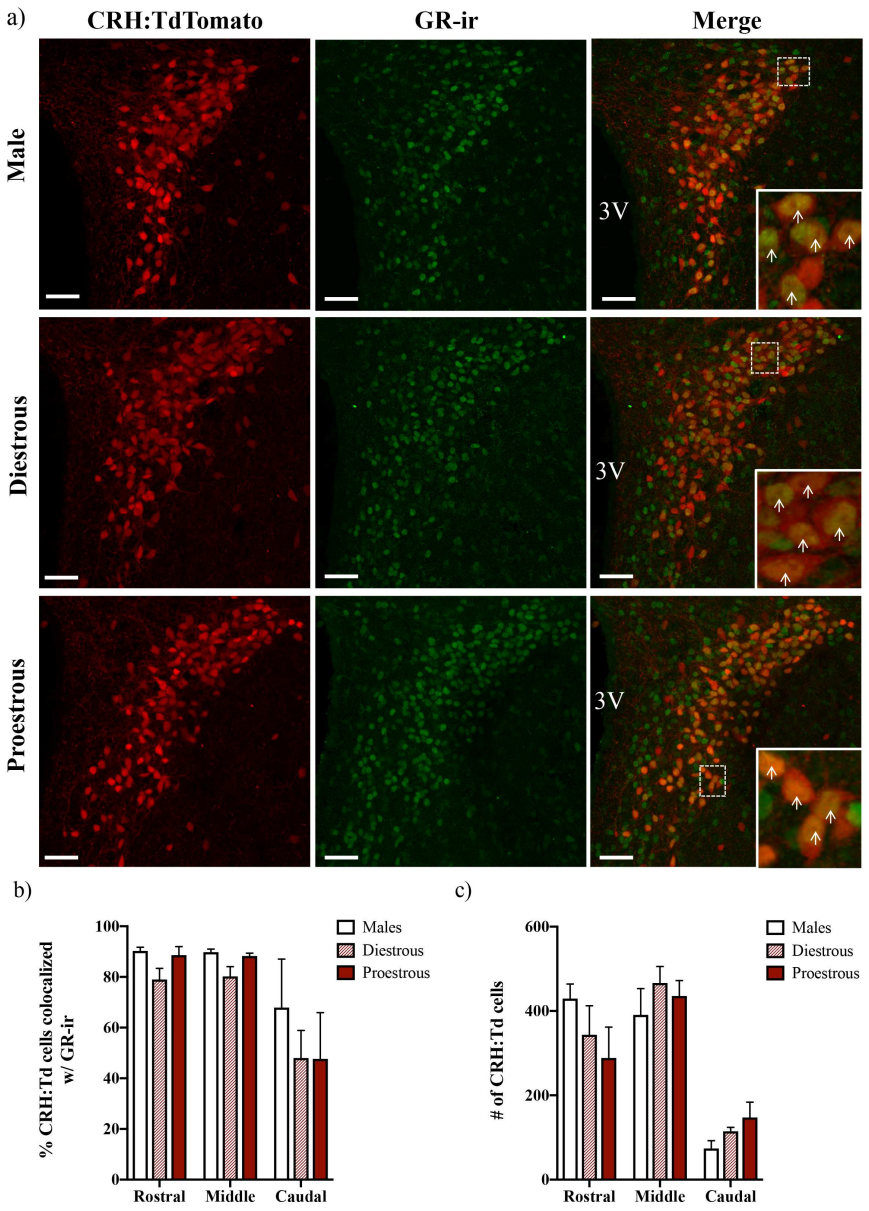

Figure 3.2. Glucocorticoid receptor (GR) expression in corticotropin releasing hormone (CRH) neurons. Representative photomicrographs of brain sections taken from *CRH-IRES-cre;Ai14* male, diestrous female, and proestrous female mice containing the rostral paraventricular nucleus (PVN; Bregma -0.83 mm) and immunolabeled for GR are shown in (a). The images on the left show CRH:TdTomato neurons in the PVN, the middle panels show GR immunoreactivity (ir), and the right panels show merged images at 26X magnification. Magnified insets highlight co-expression of CRH:TdTomato and GR-ir in the outlined box. Arrows indicate CRH:TdTomato neurons that co-express GR-ir. Scale bars: 40 um. 3V: third ventricle. Panels b and c show the mean +/- SEM percent of CRH:TdTomato neurons that co-express MeCP2-ir (b) or number of CRH:TdTomato neurons (c) in the PVN of n=3-4 mice. Two-way repeated measures ANOVAs showed main effects of PVN level on colocalization percentages ($p<0.01$) and on the number of CRH:TdTomato cells ($p<0.001$), but not of sex/estrous stage or interaction.

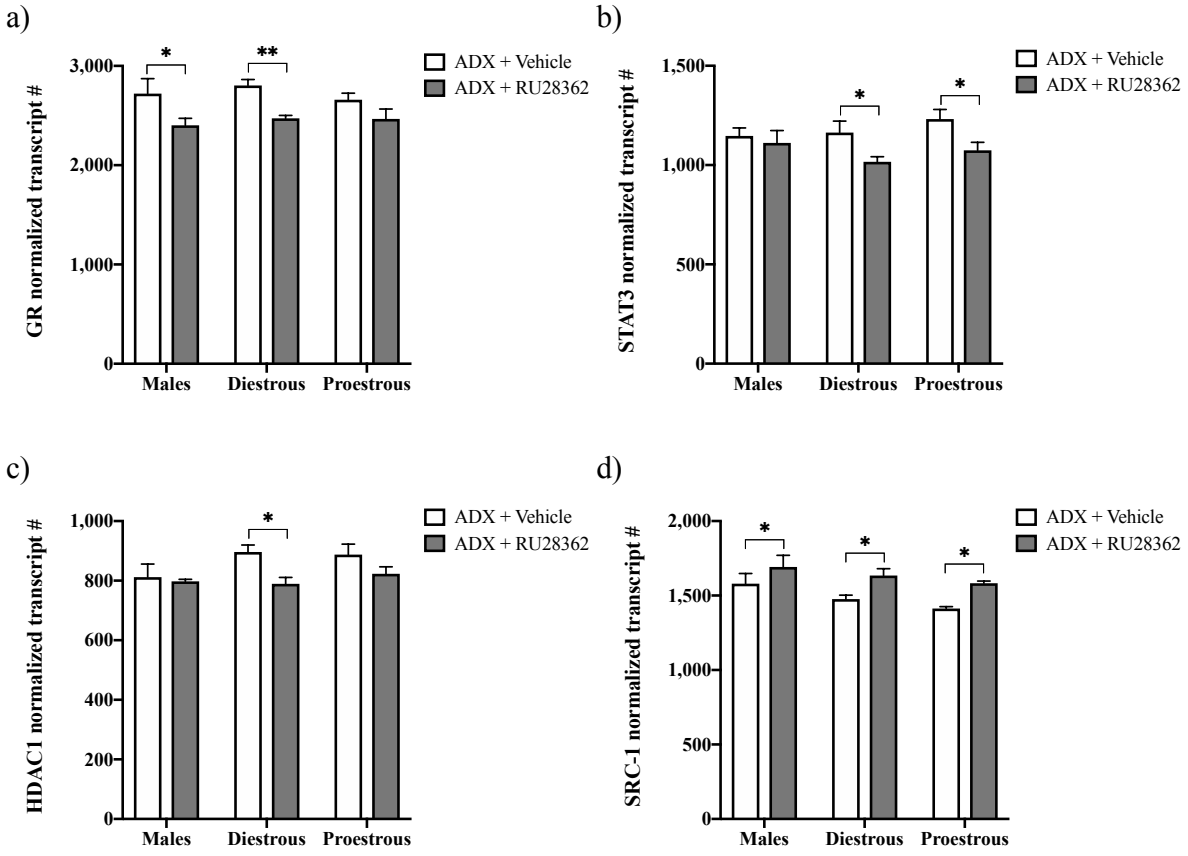

Figure 3.3. Effect of glucocorticoid receptor (GR) agonist treatment on GR and co-regulator gene expression within the paraventricular nucleus (PVN). Changes in PVN mRNAs in adrenalectomized (ADX'd) male, diestrous female, and proestrous female mice treated with RU28362 (0.4mg/kg s.c.) or vehicle are shown. mRNA levels for GR (a), signal transducer and activator of transcription 3 (STAT3; b), histone deacetylase 1 (HDAC1; c), and steroid receptor coactivator 1 (SRC-1; c) were measured using nCounter technology and normalized to the geometric mean expression of four housekeeping genes. Each bar represents mean normalized gene expression +/- SEM of n=5-7 mice. Main effects of RU28362 were found for GR ($p<0.001$), STAT3 ($p<0.01$), HDAC1 ($p<0.05$), and SRC-1 ($p<0.001$) mRNA levels by two-way ANOVAs. A main effect of sex/estrous stage was also found for SRC-1 mRNA ($p<0.05$). Planned comparisons: *=$p<0.05$ and **=$p<0.01$ for RU28362- versus vehicle-treated ADX'd male, diestrous female, or proestrous female mice.

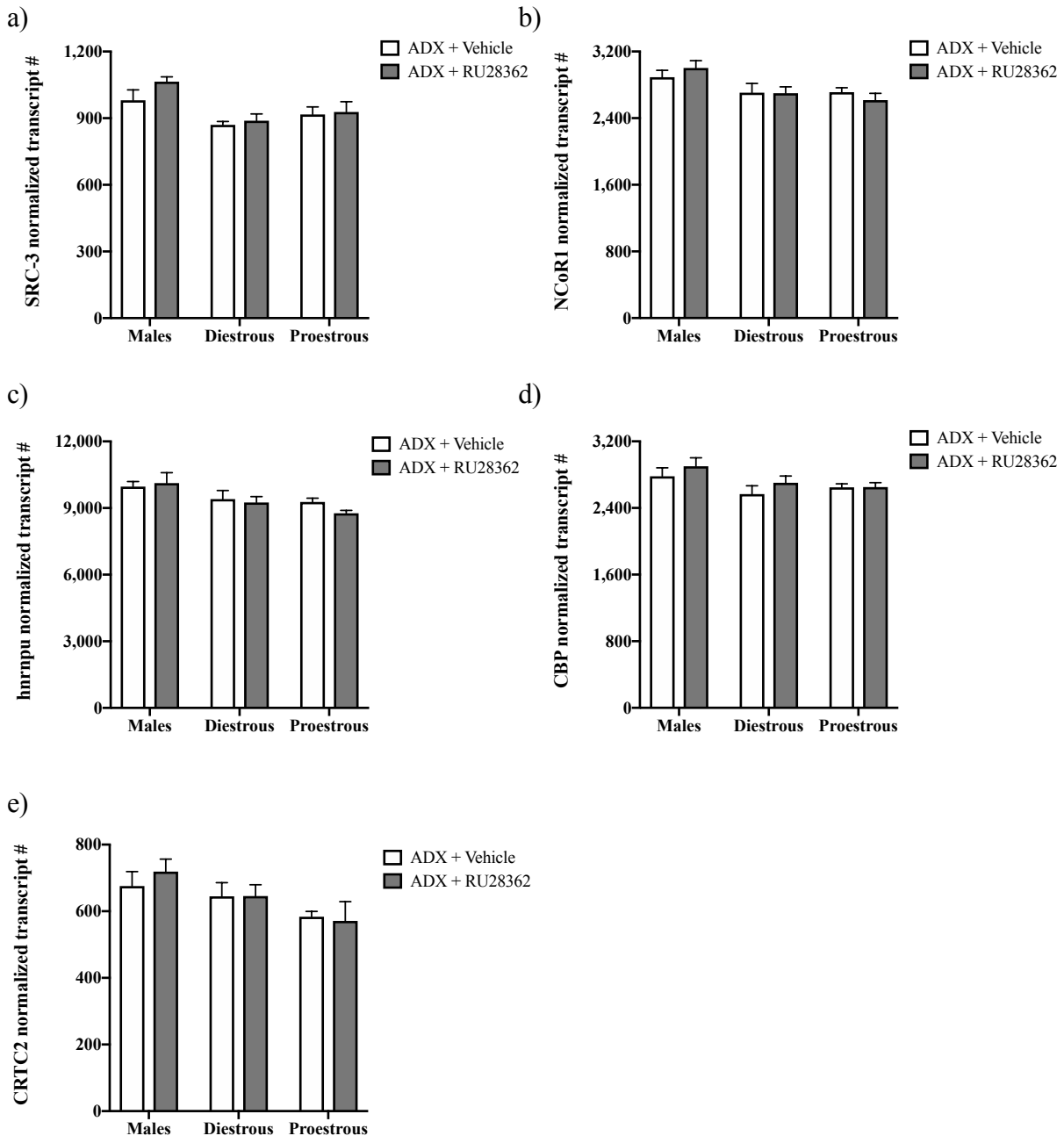

Figure 3.4. Sex/estrous stage-dependent gene expression of glucocorticoid receptor (GR) co-regulators within the paraventricular nucleus (PVN). Changes in PVN mRNAs in adrenalectomized (ADX'd) male, diestrous female, and proestrous female mice treated with RU28362 (0.4mg/kg s.c.) or vehicle are shown. mRNA levels for steroid receptor coactivator 3 (SRC-3; a), nuclear corepressor 1 (NCoR1; b), heterogeneous nuclear ribonucleoprotein U (hnrnpu; c), cAMP response element binding protein (CREB) binding protein (CBP; d), and CREB regulated transcription coactivator 2 (CRTC2; e) were measured using nCounter technology and normalized to the geometric mean expression of four housekeeping genes. Each bar represents mean normalized gene expression +/- SEM of n=4-7 mice. (a-e) Only main effects of sex/estrous stage were found by two-way ANOVAs for SRC-3 ($p<0.01$), NCoR1 ($p<0.01$), hnrnpu ($p<0.01$), CBP ($p<0.05$), and CRTC2 ($p<0.05$) mRNA levels.

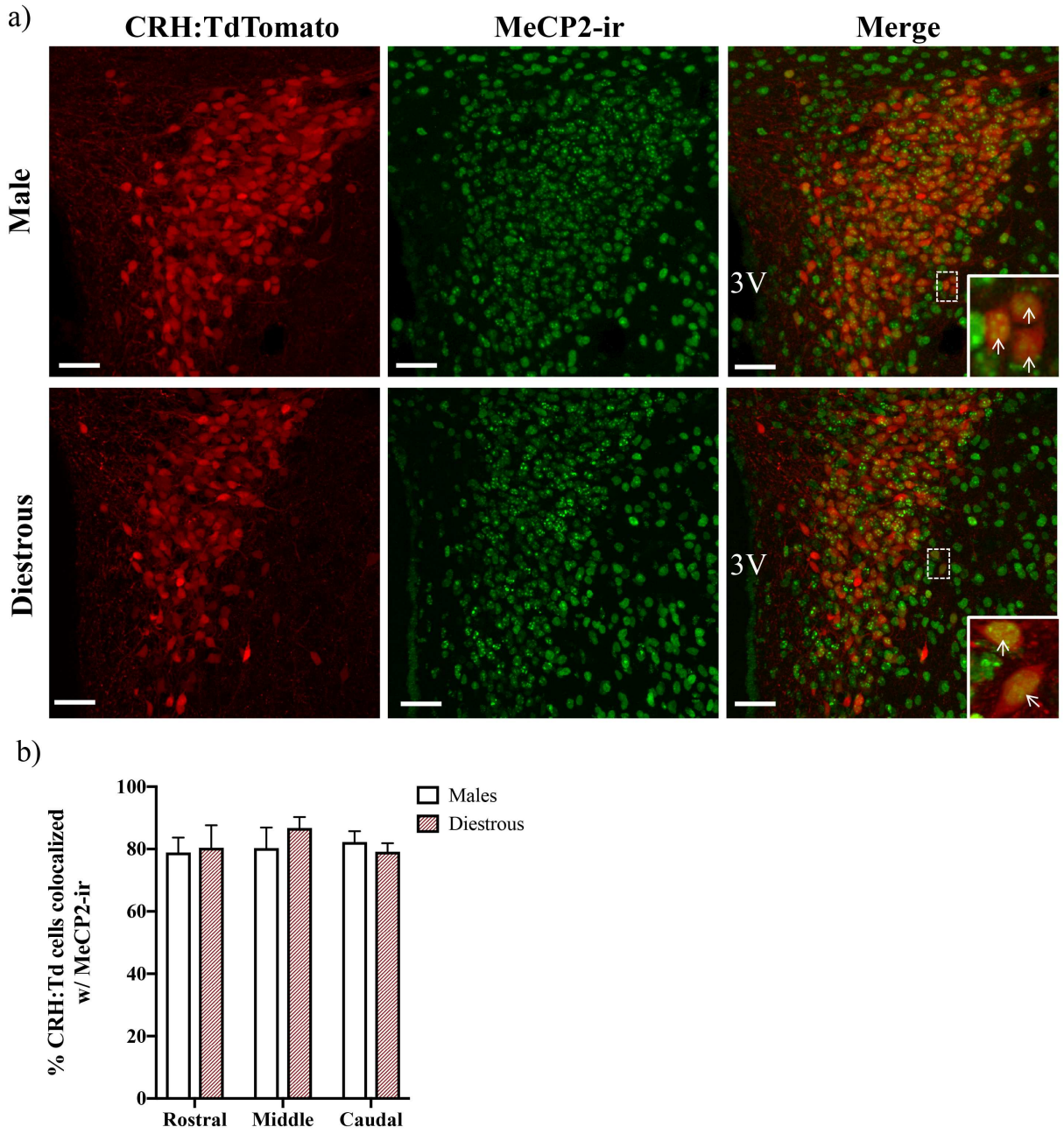

Figure 3.5. Methylated CpG binding protein 2 (MeCP2) expression in corticotropin-releasing hormone (CRH) neurons. Panel A shows representative photomicrographs of brain sections taken from *Crh-IRES-Cre;Ai14* male and diestrous female mice containing the middle paraventricular nucleus (PVN; Bregma -0.95 mm) and immunolabeled for MeCP2. The images on the left show CRH:TdTomato neurons in the PVN, the middle panels show MeCP2 immunoreactivity (ir), and the right panels show merged images at 26X magnification. Magnified insets highlight co-expression of CRH:TdTomato and MeCP2-ir. Arrows indicate CRH:TdTomato neurons that co-express MeCP2-ir. Scale bars: 40 um. 3V: third ventricle. Panel B shows the mean +/- SEM percent of CRH:TdTomato neurons that co-express MeCP2-ir throughout the PVN of n=3 mice.

3.5 Discussion

Despite the sex-related risks for psychopathologies and their correlation with sex differences in HPA axis activity (Bangasser and Valentino, 2014), few studies have examined GC regulation of the HPA axis in both sexes. In the present studies, we demonstrated a striking sex difference in GR-mediated negative feedback on PVN *Crh* expression. Whereas ADX'd male mice exhibited a small non-statistically significant decrease in CRH mRNA after two days of GR agonist treatment, ADX'd diestrous females showed a more robust and highly significant decrease. When examined on proestrus, however, ADX'd females showed an absence of a response of CRH mRNA. Interestingly, this sex/estrous stage-dependent difference was unique to *Crh* expression, as GR agonist administration decreased AVP mRNA regardless of sex or estrous stage. Although PVN CRH neurons co-expressed GRs abundantly in males, diestrous females, and proestrous females, GR, STAT3, and HDAC1 mRNAs exhibited sex/estrous stage-dependent regulation by GR agonist treatment. SRC-3, NCoR1, hnrnpu, CBP, and CRTC2 mRNAs also all were lower in diestrous and proestrous females than in males. Conversely, diestrous females and males had similar PVN MeCP2 mRNA levels, as well as similar percentages of MeCP2 co-expressing PVN CRH neurons. Thus, taken together, our findings suggest that sex differences in GR-mediated negative feedback on PVN *Crh* may depend on sex differences in the GR transcriptional machinery and an underlying influence of circulating E2 levels in females.

Previous studies have only begun to examine sex differences in GR-mediated negative feedback on PVN *Crh,* even though sex differences have been found at all levels of the HPA axis (Heck and Handa, 2019a). In a recent study, Solomon *et al*. demonstrated that PVN-selective GR knockdown results in increased adrenocorticotropic hormone (ACTH) and CORT responses to

acute stress in male mice, but not females, suggesting that PVN GR may only be necessary for feedback inhibition of the stress-activated HPA axis in males (Solomon *et al*, 2015). However, Solomon *et al*. used randomly cycling female mice, which leaves open the possibility that fluctuating E2 levels alter the GR's involvement (Solomon *et al*, 2015). Our finding that ADX'd females exhibited a robust decrease in PVN CRH mRNA on diestrus that was absent on proestrus supports this possibility. Notably, Solomon *et al*. also did not observe effects of PVN GR depletion on basal or stress-induced CRH and AVP mRNA in either sex (Solomon *et al*, 2015). As RU28362 has previously been shown to decrease PVN CRH mRNA in ADX'd male rats (Albeck *et al*, 1994), corresponding to our present findings in mice, this discrepancy likely reflects differences in experimental approaches. The GR knockdown technique employed by Solomon *et al*. involved crossing transgenic mice such that GR was depleted in the PVN throughout development and into adulthood (Solomon *et al*, 2015). Thus, consequences of GR depletion during key developmental windows that greatly influence the activity of the HPA axis in adulthood (Green and McCormick, 2016; Romeo, 2018) could help explain inconsistencies between our findings and those of Solomon *et. al* (Solomon *et al*, 2015).

Nonetheless, we observed a robust decrease in PVN CRH mRNA in ADX'd diestrous female mice following GR agonist treatment that was absent in males. The response in female mice was largely driven by greater expression of CRH mRNA in vehicle-treated ADX'd diestrous female versus male mice, suggesting that the response to ADX may be sex-dependent. In a recent study, we demonstrated that male mice exhibit a more rapid response to ADX than do diestrous female mice, as increased CRH mRNA levels measured by in situ hybridization (ISH) were found two days after ADX in males, but not diestrous females (Heck and Handa, 2019b). Although CRH mRNA was significantly increased four days after ADX in both sexes, no

statistical comparisons between sexes could be made, as males and females were run in separate ISH assays (Heck and Handa, 2019b). Consequently, the possibility remains that females have a greater increase in *Crh* expression post ADX than do males, which enables their robust *Crh* response to GR agonist treatment. While ADX'd male mice showed a small decrease in CRH mRNA following short term RU28362 administration in the present studies, it did not reach statistical significance, unlike the findings of a previous study in male rats (Albeck *et al*, 1994). Such discrepancies between our findings and those of previous studies (Albeck *et al*, 1994) could be related to the use of mice versus rats or insufficiencies in the timing and/or dose of RU28362 in our studies. However, we observed a significant effect of RU28362 that led to increased expression of *Per1,* a gene previously shown to be stimulated by GCs, in males (Balsalobre *et al*, 2000; So *et al*, 2009). We also observed a reduction in AVP mRNA following GR agonist treatment of ADX'd males and females. Thus, the dose of RU28362 was sufficient for regulation of *Avp*, but not *Crh*, in both sexes, suggesting that males may ultimately rely more on GR regulation of PVN *Avp* versus *Crh* to limit neuroendocrine stress responses. Accordingly, in male rats, PVN AVP mRNA levels have been suggested to be more sensitive to GC negative feedback than are levels of CRH mRNA (Makino *et al*, 1995b).

Interestingly, the sex difference in GR regulation of PVN *Crh* may only hold true when females have low E2 levels. As in males, no significant effect of GR agonist treatment on PVN CRH mRNA was observed in ADX'd proestrous females. That females can have varying sensitivity to *Crh* regulation by the GR depending on their estrous cycle stage is well supported by evidence that the estrous cycle alters the activity of the HPA axis (Heck and Handa, 2019a). Increasing E2 concentrations throughout the estrous cycle have been shown to be directly proportional to the basal and stress-induced activity of the HPA axis. Accordingly, female

rodents on diestrus (low E2) have low basal GC secretion and a relatively quick on-off response to stressors, whereas females on proestrus (high E2) have elevated basal GC levels and a prolonged GC response to stressors (Carey *et al*, 1995; Herman *et al*, 2016; Viau and Meaney, 1991). Importantly, elevations in HPA activity are greatest on proestrous morning, when E2 levels are peaking, but elevations in progesterone have not yet occurred, as progesterone appears to inhibit E2's effects on HPA output (Viau and Meaney, 1991). Because we examined female mice no later than 1400 h on proestrus, it is likely that the changes in the *Crh* response to RU28362 we observed on proestrus versus diestrus are due to E2 rather than progesterone. Supporting this possibility, E2 has been shown to inhibit GC negative feedback on PVN CRH in female rats (Weiser and Handa, 2009).

Notably, androgens may also influence GR-mediated negative feedback on PVN *Crh* to drive sex differences, as we previously demonstrated an effect for the potent androgen, dihydrotestosterone, to facilitate the CRH mRNA response two days after ADX in males (Heck and Handa, 2019b). Although it does not appear that gonadal hormones influence the response of PVN CRH mRNA four days after ADX, as both male and female mice exhibited increased CRH mRNA following ADX alone or ADX paired with gonadectomy (Heck and Handa, 2019b), the possibility remains that androgens influence the effects of the GR on *Crh* expression. The upregulation of Per1 mRNA in the PVN of ADX'd males, but not diestrous or proestrous females, we observed after RU28362 treatment supports the possibility that the presence of androgens in males amplifies some of the actions of the GR. Moreover, this effect of androgens, which may contribute to sex differences in GR sensitivity, also appears to be gene specific, since there was no sex effect on the ability of RU28362 to decrease AVP mRNA expression.

Despite the sex and estrous stage-related response of *Crh* to GR regulation, male, diestrous female, and proestrous female mice all exhibited co-expression of the GR in almost all CRH neurons within the rostral and middle regions of the PVN. In the mouse, the anterior two-thirds of the PVN contains the vast majority of neuroendocrine neurons that regulate the HPA axis, whereas the posterior one-third mostly contains preautonomic neurons that coordinate autonomic outflow (Biag *et al*, 2012). The greater percentage of CRH:TdTomato and GR-ir colocalization we observed in the rostral/ middle versus caudal PVN may, therefore, be related to the predominant role the rostral/ middle PVN plays in regulating the HPA axis. However, the lesser colocalization in the caudal PVN may also simply reflect the presence of significantly fewer CRH:TdTomato neurons within the region.

Although abundant GR co-expression in PVN CRH neurons has previously been reported in male rats (Uht *et al*, 1988), subjects were ADX'd in that study to overcome the technical hurdle of capturing the entire population of CRH neurons using immunohistochemical approaches. CRH is readily downregulated by endogenous GCs, making this difficult without such manipulation (Kovács, 2013). In the present studies, we utilized *CRH-IRES-Cre;Ai14* mice, which provide the unique advantage of permanently labeling all CRH expressing neurons without the need for non-physiological manipulations, such as ADX or colchicine for complete visualization. In these mice, the *Crh* promoter drives expression of cre recombinase, which in turn, induces the expression of a TdTomato reporter through the removal of a loxp-STOP-loxp site upstream of the TdTomato reporter. TdTomato expression is then driven by a CAG promoter and permanently marks CRH neurons. Importantly, TdTomato fluorescence highly overlaps with CRH protein (Smith *et al*, 2014; Wamsteeker Cusulin *et al*, 2013) in the PVN but not with other neuropeptide neurons characteristic of the region (Wamsteeker Cusulin *et al*, 2013). TdTomato

also reliably targets stress-responsive neuronal populations in the PVN (Wamsteeker Cusulin *et al*, 2013). Thus, our GR and CRH colocalization findings are novel in the use of the *CRH-IRES-Cre;Ai14* mouse model, as well as in their incorporation of both male and female subjects.

While the localization of GRs in CRH neurons supports their involvement in a direct mechanism of *Crh* regulation in both sexes, further studies are necessary to determine if ADX and/or GR agonist treatment influence GR/ CRH colocalization in a sex and/or estrous stage-dependent fashion, as GRs are also subject to downregulation by GCs (Makino *et al*, 1995b; Patchev *et al*, 1994a). Then again, GR gene expression was similar in the PVN of vehicle-treated ADX'd male, diestrous female, and proestrous female mice. The lack of sex/estrous stage-related differences in both CRH/ GR colocalization and GR mRNA levels of vehicle-treated mice in the present studies is perhaps surprising, given evidence of lower GC binding in the hypothalamus of randomly cycling female versus male rats (Turner and Weaver, 1985) and that E2 treatment can down-regulate GR in female rat hypothalamus (Burgess and Handa, 1993). These previous studies suggest that females have fewer hypothalamic corticosteroid receptors. However, they used randomly cycling females (Turner and Weaver, 1985) or ovariectomized females treated with E2 (Burgess and Handa, 1993) and did not measure GR in the PVN selectively. While it is possible that sex/estrous stage-related changes in GR gene expression exist within PVN CRH neurons, a more quantitative and anatomically selective approach is necessary to assess this. An alternative possibility is that sex differences are present in GR function, rather than numbers, reflecting other sex/estrous stage-reliant molecular mechanisms downstream of receptor binding.

In this regard, we also examined sex/estrous-dependent effects of RU28362 treatment on the expression of a putative GR-DnMT3b-MeCP2-HDAC1 repressor complex, as well as SRC-1, both of which have been proposed to negatively regulate *Crh* (Lachize *et al*, 2009; Sharma *et al*,

2013). Specifically, whereas SRC-1 mRNA levels were significantly increased following RU28362 treatment in ADX'd mice, irrespective of sex and estrous stage, GR and HDAC1 mRNAs exhibited sex/estrous stage- dependent regulation by RU28362. GR mRNA was decreased in males and diestrous females, but not proestrous females, supporting the possibility that increasing E2 levels inhibit GR's autologous regulation, as previously reported in the hypothalamus (Burgess and Handa, 1993), to influence sex differences in GR-mediated negative feedback on PVN *Crh*. HDAC1 mRNA, alternatively, was decreased by RU28362 exclusively in diestrous females, again supporting a role for increasing E2 to inhibit the function of an HDAC1-containing repressor complex and thereby influence sex-based *Crh* expression. Further studies are necessary to determine if and/or how decreases in GR and HDAC1 expression correspond to changes in *Crh* expression. Nonetheless, such changes support the possibility of sex/estrous stage-dependent involvement of a GR-DnMT3b-MeCP2-HDAC1 repressor complex in GR-mediated negative feedback on PVN *Crh*. Our findings similarly support the sex-dependent involvement of STAT3, a coregulator previously associated with GR regulation outside the context of PVN *Crh* (Petta *et al*, 2016), as STAT3 mRNA levels were decreased by RU28362 in both ADX'd diestrous and proestrous females, but not in males. However, an important caveat to consider here and throughout this discussion is that we cannot restrict our gene expression findings to only CRH neurons in the PVN. Hence, any of the changes in the gene expression of GR-associated transcriptional regulators could ultimately alter the expression of other GR-regulated genes, or other genes outside of GR regulation altogether.

Although we did not observe an effect of RU28362 on PVN MeCP2 mRNA in ADX'd subjects, we also chose to examine expression of MeCP2 in CRH neurons of the male and female mouse PVN, under the assumption that MeCP2 can be found in many neuropeptide

neurons of the PVN. In addition to its role in a putative GR-DnMT3b-MeCP2-HDAC1 repressor complex (Sharma *et al*, 2013), MeCP2 has been implicated in *Crh* transcriptional repression *in vivo* (McGill *et al*, 2006). Accordingly, increased PVN CRH mRNA and decreased MeCP2 binding to the *Crh* promoter have been shown in mice expressing a mutated MeCP2 protein (McGill *et al*, 2006). Thus, MeCP2 is thought to be central to *Crh* down-regulation, and further assessment of its sex-dependent involvement in our studies was warranted. We found that most PVN CRH:TdTomato neurons co-expressed MeCP2-ir in male and diestrous female *CRH-IRES-Cre;Ai14* mice, supporting its involvement in *Crh* regulation of mice of both sexes. Future studies using single cell analysis to quantify MeCP2 expression in PVN CRH neurons and examine sex/estrous stage-dependent differences will further enhance our understanding of its potentially sex-dependent involvement in the GR's regulation of *Crh*.

Sex-dependent expression of GR transcriptional co-regulators, including SRC-3, NCoR1, hnrnpu, CBP and CRTC2 mRNAs, was also found in ADX'd subjects, supporting the possibility of their sex-dependent involvement in GR-mediated negative feedback on PVN *Crh*. Although roles for SRC-3 and hnrnpu in GR's regulation of PVN *Crh* have not yet been identified, both proteins have been associated with GR's regulation of other genes (Eggert *et al*, 1997; Rollins *et al*, 2015). A role for CBP and CRTC2, on the other hand, has been better established. Jeanneteau *et al*. proposed a mechanism of negative feedback in which GR signaling opposes the excitatory influence of BDNF-TrkB-CREB signaling on PVN *Crh* (Jeanneteau *et al*, 2012). Thus, CBP and CRTC2, as components of the excitatory TrkB-CREB signaling cascade, may be decreased in ADX'd females to shift the balance in *Crh* regulation toward the GC influence. This may contribute to the greater GR inhibition of *Crh* evident in diestrous females versus males. A role for NCoR1 has also been identified in the repression of CREB-mediated *Crh* induction in a cell

model system for *Crh* repression by GCs (Van Der Laan *et al*, 2008), and it may be decreased in ADX'd females to further alter the balance between CREB and GC signaling at the *Crh* promoter.

Taken together, our findings have demonstrated a pronounced sex difference in GR-mediated negative feedback on PVN *Crh* that may rely on sex differences in the GR transcriptional machinery, as well as an underlying influence of estrous cycle stage in female mice. Using a novel NanoString nCounter technology, we observed significant changes in mRNAs for the GR and its transcriptional co-regulators that might have otherwise been undetectable. This technology offers the unique advantage of quantifying expression of multiple mRNAs with great accuracy and sensitivity directly from total RNA without the need for reverse transcription and amplification, steps which can add variance (Veldman-Jones *et al*, 2015). Although we cannot definitively say that the sex-, estrous cycle- and GC- dependent changes in the transcriptional regulator mRNAs we identified influence GR's repression of *Crh*, these findings will help guide future investigations of the sex-dependent mechanisms of GC negative feedback on the HPA axis.

Chapter 4: Chronic variable stress alters hypothalamic-pituitary-adrenal axis function in the female mouse[5]

4.1 Summary

Chronic stress is often associated with a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, which can greatly increase risk for a number of stress-related diseases, including neuropsychiatric disorders. Despite a striking sex-bias in the prevalence of many of these disorders, few preclinical studies have examined female subjects. Hence, the present study aimed to explore the effects of chronic stress on the basal and acute stress-induced activity of the HPA axis in the female C57BL/6 mouse. We used a chronic variable stress (CVS) paradigm in these studies, which successfully induces physiological and behavioral changes that are similar to those reported for some patients with mood disorders. Using this model, we found pronounced, time-dependent effects of chronic stress on the HPA axis. CVS-treated females exhibited adrenal hypertrophy, yet their pattern of glucocorticoid secretion in the morning resembled that of controls. CVS-treated and control females had similar morning basal corticosterone (CORT) levels, which were both significantly elevated following a restraint stressor. Although morning basal gene expression of the key HPA-controlling neuropeptides corticotropin releasing hormone (CRH), arginine vasopressin (AVP) and oxytocin (OT) was unaltered within the paraventricular nucleus (PVN) by CVS, CVS altered the PVN OT and AVP mRNA responses to acute restraint. In control females, acute stress decreased AVP, but not OT mRNA; whereas, in CVS females, it decreased OT, but not, AVP mRNA. Unlike the morning pattern of HPA activity, in the evening, CVS-treated females showed increased basal CORT with

[5]Chapter 4 is modified from: Chronic variable stress alters hypothalamic-pituitary-adrenal axis function in the female mouse. Physiology & Behavior. Vol. 209, October 1, 2019.

hypoactive responses of CORT and PVN c-Fos immunoreactivity to restraint stress. Furthermore, CVS elevated evening PVN CRH and OT mRNAs in the PVN, but it did not influence anxiety- or depressive-like behavior after a light/dark box or tail suspension test. Taken together, these findings indicate that CVS is an effective model for HPA axis dysregulation in the female mouse and may be relevant for stress-related diseases.

4.2 Introduction

The allostatic load hypothesis posits that an organism's repeated efforts to maintain homeostasis in response to chronic exposure to stressors induce a state of dysregulation referred to as an "allostatic state," which can have important health consequences. One common consequence is increased susceptibility to mood disorders such as anxiety and depression (McEwen, 2003). Indeed, chronic interpersonal stress increases the risk of developing depression (Vrshek-Schallhorn *et al*, 2015), and stressful and negative life events are associated with adverse symptom trajectories for individuals with anxiety or depression (Nandi *et al*, 2009). Many of these disorders are more prevalent in women relative to men, even after controlling for social and cultural effects (Bekker and van Mens-Verhulst, 2007; Piccinelli and Wilkinson, 2000). Despite this sex-bias, the vast majority of preclinical research modeling mood disorders has focused exclusively on male subjects (Palanza, 2001), leaving a critical need for further investigation of sex-specific effects within mood disorder models.

The allostatic load hypothesis has been modeled using a chronic variable stress (CVS) paradigm, which exposes rodents to a variety of mild stressors applied in an unpredictable manner over a prolonged period of time (Willner *et al*, 1987). This model reliably induces physiological and behavioral changes that are similar to those in patients with mood disorders, and shows high predictive validity in responsiveness to antidepressant medication (Willner,

2017). CVS has also been found to reproduce the hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis observed in some patients with mood disorders (Swaab *et al*, 2005), as indicated by upregulated corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) mRNAs within the paraventricular nucleus (PVN) of the hypothalamus coupled with elevated plasma corticosterone (CORT) levels (Franco *et al*, 2016; Liu *et al*, 2017; Meynen *et al*, 2006). Although the source of altered HPA axis function following CVS is not well-characterized, the hormone oxytocin (OT) is an attractive target for alteration following chronic stress exposure. In addition to this neuropeptide's mediation of HPA axis function (Neumann, 2002), OT mRNA has been shown to be elevated in patients with mood disorders (Dai *et al*, 2017). It has also been reported to be increased (Zheng *et al*, 2010) or decreased (Flak *et al*, 2011) by CVS in the male rat.

Some evidence suggests that sex may influence the effects of CVS on both HPA axis function and affective behavior in the rat (Duncko *et al*, 2001; Vieira *et al*, 2018), but the consequences of CVS exposure for the female mouse are less understood. Thus, the present set of experiments sought to investigate the impact of CVS on female HPA axis function in the C57BL/6 mouse. These studies aimed to first identify consequences of CVS on basal and stress-induced activity of the HPA axis. Because of known circadian rhythms in the activity of the HPA axis, we examined HPA function in both the morning and evening (Dallman *et al*, 1987; Kwak *et al*, 1993; Maejima *et al*, 2017; Muglia *et al*, 1997; Nicolaides *et al*, 2014; Watts *et al*, 2004). Secondly, these studies sought to characterize the effects of CVS on neuropeptide expression and anxiety- and depressive-like behavior in the female mouse.

4.3 Materials and Methods

Subjects

Two-month-old female C57BL/6 mice were purchased from Charles Rivers Laboratories (Wilmington, MA) and maintained on a 12:12 light cycle (lights on at 0600 h) in the Colorado State University Laboratory Animal Research facility. Subjects were pair-housed in one of two colony rooms and access to food and water was available *ad libitum*. All animal protocols were approved by the Colorado State University Institutional Animal Care and Use Committee and were performed in accordance with the guidelines of Colorado State University, the National Institutes of Health, and the Association for Assessment and Accreditation of Laboratory Animal Care International.

To control for potential effects of changing gonadal hormone levels across the estrous cycle, all female subjects were examined outside of the proestrous phase of the cycle when estradiol levels are highest (Butcher *et al*, 1974). Estrous cyclicity was monitored for approximately two weeks prior to and on the day of sacrifice by daily vaginal lavage (collected between 0900-1100 h) using a 0.9% saline solution. Samples were stained with methylene blue (0.05%) and visualized using light microscopy as previously reported (Marcondes *et al*, 2002).

Experiment 1. Effect of CVS on the morning HPA axis response to a novel, acute stressor

Adult female mice were exposed to CVS daily, over a six-week period, as previously described (Borrow *et al*, 2018). Briefly, subjects experienced an average of two stressors per day, administered at variable time points. Stressors included three hours of occupying a cage with damp bedding, no bedding, or bedding soiled by same-sex unfamiliar mice, three hours of cage tilt (approximately 45°), one hour of exposure to cat odor, eight hours of white noise (85 dB) and overnight exposure to overhead light. Control subjects were housed in a separate colony room to

prevent unintended stress exposure. For more details on the protocol used for CVS, see Tables 4.1 and 4.2.

After six weeks of CVS, CVS-treated (n=9) and control (n=9) subjects were exposed to 20 minutes of restraint stress in closed, ventilated conical tubes inside their home cages between 0900 and 1100 h or were left unperturbed. Subjects were anesthetized with isoflurane, weighed, and decapitated immediately following the restraint stress. Nonstressed animals were killed within one minute of first disturbance of their home cage. Upon decapitation, trunk blood was collected from all animals into chilled tubes containing 0.5 M ethylenediaminetetraacetic acid and aprotinin (4 mg/ml; Sigma-Aldrich, St. Louis, MO) and centrifuged at 3000 rpm for 12 minutes in a Beckman J6 centrifuge. Plasma was isolated and stored at −20°C until assayed for CORT and OT by radioimmunoassay (RIA). Brains and adrenal glands were also collected shortly following decapitation, flash-frozen and stored at −80°C.

Table 4.1. CVS stressors
* Restraint stress was excluded from experiment 1.

Stressor	Description
Social stress	Subjects are placed in an empty cage previously occupied by novel female mice for 3 h
Restraint stress	Subjects are placed in a closed, ventilated conical tube for 30 min*
Novel object exposure	Novel objects (8 marbles) are placed in each cage overnight
Damp bedding	125 mL of water is added to subjects' cages. Subjects remain in cage with damp bedding for 3 h before being placed in a new cage
Empty cage	Subjects are placed in a new cage without sawdust bedding for 3 h
Light cycle disturbance	Subjects are exposed to continuous overhead light for 36 h
Noise exposure	Subjects are exposed to a pink noise (80 dB) for 8 h
Cage tilt	Cages are tilted backward at a 45° angle for 3 h
Predator odor	Subjects are exposed to cat odor (10 mL of soiled cat litter in 15 mL conical tubes punctured with small holes to allow odor dispersion) for 60 min

Table 4.2. Representative timelines for stressors during the six-week CVS protocols. Stressors 1 and 2 were administered at varying time points before or after 1200 h, respectively. D= damp bedding; E= empty cage; L= light cycle disturbance; M= novel object exposure; N= noise exposure; O= predator odor; R= restraint stress; S= social stress; T= cage tilt.

Experiment 1

Day:	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Stressor 1:	D	S		T		N		S	S	D	T	D	T	
Stressor 2:	E	M	O	L	L		D	M	T	O	L	M	L	M
Day:	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
Stressor 1:	E	T	D	T	E			S	D	T	D	T		
Stressor 2:	M	L	S	M	O	N	D	N	O	M	N		M	D
Day:	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**
Stressor 1:	T	E	D		T			T	D		E	E		M
Stressor 2:	S	M	O	L	M	O	N	L	O	L	M	T	N	L

Experiment 2

Day:	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Stressor 1:	E	R	T	D	E	T	E	R	D	S	R	D	E	T
Stressor 2:	L	O	N	L		M		O	N	M	T	L		M
Day:	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
Stressor 1:	D	R	S	T	E	S	T	D	E	T	R	S	E	O
Stressor 2:		L	N	O	M		O	L	M	N		D	L	N
Day:	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**
Stressor 1:	S	R	D	S	T	E	S	R	O	T	E		R	
Stressor 2:	T		N	L		M		D	L	S	N	M	L	N

Experiment 2. Effect of CVS on the evening HPA axis response to an acute stressor

Adult female mice were exposed to CVS or control conditions daily, over a six-week period as in Experiment 1. However, in Experiment 2, the stressors presented twice-daily included 30 minutes of restraint stress in addition to those listed above (Tables 4.1 and 4.2). CVS-treated (n=8) and control (n=6-7) subjects were exposed to 20 minutes of restraint stress in closed, ventilated conical tubes in their home cages or were left undisturbed in their home cages approximately one hour prior to dark cycle onset (1700 h). Trunk blood was collected immediately after the 20-minute restraint stressor, and plasma was isolated and stored until it was assayed for CORT by RIA as in Experiment 1.

A separate cohort of CVS-treated (n=8) and control (n=8) subjects were examined for stressor-induced neuronal activation in the PVN. Subjects were exposed to 20 minutes of restraint stress within closed, ventilated conical tubes in their home cages at 1700 h (lights off at 1800 h). Animals were released back into their home cages until approximately 90 minutes after onset of the restraint stress, at which point they were anesthetized with isofluorane and transcardially perfused with 0.1M phosphate buffered saline followed by 4% paraformaldehyde for measurement of c-Fos.

Experiment 3. Effect of CVS on anxiety- and depressive-like behavior and on PVN neuropeptide gene expression

As in Experiment 2, female mice were exposed to a six-week CVS protocol. At the end of six weeks of CVS, CVS-treated (n=8) and control (n=7) subjects were habituated to a behavioral testing room in the afternoon (1400-1600h) for 30 minutes prior to a light/dark box test. The apparatus (40 cm x 40 cm) consisted of two chambers separated by a barrier with a door permitting entry between chambers. The light chamber was illuminated to 2000 lm, while the dark chamber was measured at approximately 10 lm. Each subject was placed in the light

chamber facing the doorway, and behavior was video recorded (Bunker Hill Security) for five minutes. Videos were scored by an experimenter blind to treatment condition. All testing occurred between 1400-1600 h. The apparatus was sanitized before and after each subject using a 70% ethanol solution.

A separate group of CVS-treated (n=11) and control (n=7) subjects were habituated to a behavioral testing room for 30 minutes prior to a tail suspension test. Each subject was suspended from a horizontal bar for ten minutes by its tail from a height of 12 inches with a section of tape while behavior was video recorded. All testing was conducted between 1400-1600 h. Given the tendency of C57BL/6 mice to climb their tails during this test, climbing was prevented by slipping a short segment from a plastic straw over each subject's tail prior to suspension. The tail suspension apparatus was sanitized before and after each subject using a 70% ethanol solution. Duration of immobility was subsequently scored from video footage by an experimenter that was blind to treatment group.

Two hours after testing in the light/dark box or tail suspension test, CVS-treated (n=6) and control (n=6) subjects were anesthetized with isoflurane and decapitated. Brains were flash-frozen in 2-methylbutane (-40°C) and stored at -80°C. Brains were used for droplet digital PCR (ddPCR) to examine neuropeptide gene expression in the PVN.

Radioimmunoassays (RIAs)

Plasma CORT was measured as previously described. Briefly, plasma was diluted (1:25) in PBS and plasma binding proteins were denatured by heating to 65°C for one hour. Diluted plasma samples were incubated overnight at 4°C with rabbit anti-CORT antiserum (1:1200, MP Biomedicals, Sonon OH) and 3H-CORT (PerkinElmer, Boston, MA) in 0.01 M PBS containing 0.1% gelatin. Dextran coated charcoal was used to separate antibody bound CORT from free

CORT. Standard curves were constructed from dilutions of CORT (4-pregnen-11β, 21-diol-3, 20-dione; Steraloids, Wilton, NH; 5–500 ng/ml). For all assays, the intra-assay coefficient of variation was less than 10%.

To measure circulating OT levels, plasma was subjected to an extraction protocol that was adapted from a previous report using methanol (Moenter *et al*, 1991). Briefly, 2.0 ml methanol was added to all samples and, following vortex and centrifugation, the supernatant was further subjected to cold precipitation (–20°C) for 24 hours. After three rounds of cold precipitation, the supernatant was dried under nitrogen at 45°C and resuspended in RIA buffer to the same volume as the starting amount. This product was then used in the protocol for an OT RIA kit (RK-051-01; Phoenix Pharmaceuticals, Burlingame, CA) following manufacturer's instructions. The extraction procedure resulted in the recovery of 69.1% of the starting material as determined by spiking samples with known amounts of 125I-oxytocin. The intra-assay coefficient of variation was 4.5%.

In situ hybridization

16-µm brain sections were cut in the coronal plane using a CM3050 S cryostat (Leica, Wetzlar). Sections containing the PVN were cut at –20°C, thaw-mounted onto positively charged slides (Superfrost Plus, VWR Scientific, West Chester, PA), and stored at -80°C. In situ hybridization was performed as previously described (Borrow *et al*, 2018). Briefly, tissue was thawed to room temperature, fixed, acetylated, delipidated, dehydrated in graded ethanols and air-dried. 48-bp oligonucleotide mRNA probes for *Crh* (5'CAGTTTCCTGTTGCTGTGAGCTTGCTGAGCTAACTGCTCTGCCCGGGC-3'), Ot (5'-AAGCAGGCAGCAAGCGAGACTGGGGCAGGCCATGGCG ATGGTGCTCAG-3') and Avp (5'GTAGACCCGGGGCTTGGCAGAATCCACGGAC TCCCGTGTCCCAGCCAG-3') were

end-labeled with [35S] using terminal deoxynucleotidyl transferase (Thermo Scientific, Waltham, MA) and added to hybridization solution at a concentration of 20 × 106 cpm/mL. Brain sections were incubated in this hybridization solution at 37°C overnight and then washed and dehydrated in a series of solutions with increasing levels of ethanol. To examine hybridization intensity, slides were exposed to X-ray film (Carestream Kodak Biomax MR, Carestream, Rochester, NY) for 4 days (*Crh*), 1 day (*Avp*), or 14 hours (*Ot*) to generate autoradiograms. Film autoradiograms were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD) to quantify optical density in 4-6 PVN containing tissue sections. The density of exposed pixels in each half of the PVN for all sections was measured using a template of fixed size and expressed as arbitrary density units (ADUs). Background density in an adjacent area without labeling was subtracted from each measurement and resulting ADUs were averaged to obtain a single value per animal for statistical analysis. All density calculations were performed by an experimenter blind to treatment condition.

Droplet digital PCR (ddPCR)

Flash-frozen brains were sectioned by cryostat (Leica CM3050 S, Leica Biosystems) at 300 μm, and the PVN was isolated via micropunch with a 0.98 mm diameter cannula. RNA extraction of PVN tissue was performed using an RNeasy mini kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Total RNA was quantitated using an Epoch Microplate Spectrophotometer and Gen5 v. 1.11 data analysis software (BioTek, Winooski, VT). cDNA was generated using an iScript cDNA synthesis kit (Bio-Rad, Munich, Germany). For each ddPCR reaction, 5 uL of template DNA was added to a master mix containing primers for CRH (F: 5'-ATGCTGCTGGTG GCTCTGTC; R: 5' GGATCAGAACCGGCTGAGGT-3') or OT (F: 5'-AAGGGAGCTGCAGTGGAGTA-3'; R: 5'-AGACTGGCAGGGCGAAG-3'),

Evagreen (Bio-Rad, Munich, Germany) and RNAse free water. 20 uL of sample and 70 uL of droplet generation oil (Bio-Rad, Munich, Germany) were pipetted into DG8 Cartridge (Bio-Rad, Munich, Germany) wells and secured with a gasket. Droplets were then generated in a QX200 droplet generator (Bio-Rad, Munich, Germany), transferred to a 96-well plate, and heat-sealed. PCR was performed in a thermal cycler (C1000 Touch, Bio-Rad, Munich, Germany) using the following protocol: 95°C for 10 minutes (1 cycle), 95°C for 30 seconds then 60°C for 1 minute (40 cycles), 4°C for 5 minutes (1 cycle), 90°C for 5 minutes (1 cycle), and hold at 4°C. The ramp rate was set at 2°C/second, the sample volume at 40 μl, and the heated lid at 105°C. After PCR amplification, droplets were analyzed in a QX200 droplet reader (Bio-Rad, Munich, Germany), and the absolute template expression in copies/ul input was quantified using QuantaSoft software (Bio-Rad, Munich, Germany). All values were normalized to the amount of cDNA loaded in each reaction, which was calculated based on cDNA concentrations quantified using the Quant-iT OliGreen ssDNA Assay Kit (Thermo Scientific, Waltham, MA) according to manufacturer's instructions.

Immunohistochemistry

Brains from perfused animals in Experiment 2 were collected and post-fixed for 24 hours in 4% paraformaldehyde at 4°C, then infiltrated with 30% sucrose at 4°C until equilibrated. Immunohistochemistry was performed on the fixed brain tissue to examine changes in PVN neuronal activation. Fixed brains were sectioned into four series of 35 μm thickness on a cryostat at −16°C, then immunohistochemistry was performed on a series of free-floating sections. Tissue was washed in 0.1 M PBS, blocked in 5% normal donkey serum (Jackson Laboratories, Bar Harbor, ME) for one hour at room temperature, then incubated overnight in a previously validated goat anti-c-Fos primary antibody (1:2000; Santa Cruz Biotechnology, Dallas TX)

(Isingrini *et al*, 2016). The next day, tissue was washed in a PBS-Triton X solution, then incubated in donkey anti-goat Alexa Fluor (AF) 568 (Jackson Laboratories, Bar Harbor, ME) for two hours at room temperature. After washing in PBS-Triton X, tissue was mounted onto glass slides and coverslipped with ProLong Gold Antifade Mountant (Thermo Fisher). Fluorescence was visualized using an LSM 880 confocal scanning microscope (Carl Zeiss, Jena, Germany) and 10X objective (Zeiss Plan-Apochromat 10X/0.45∞/0.17), and 25 um-thick Z-stacks (1 um thick optical sections) were taken through the PVN. Immunohistochemical analysis of Z-stack images was conducted using Imaris v8.0 (Bitplane, Concord, MA). For each subject, 2-3 images per hemisphere were captured for the PVN. For each image, the number of c-Fos-immunoreactivity *(ir)* expressing neurons was determined using the automated counting application in Imaris v8.0 and manually checked and scored by an investigator that was blind to treatment condition. Values from all images were averaged together for each subject.

Statistical analyses

All statistical analyses were performed using the Prism statistical program (GraphPad Software, La Jolla, CA). The effects of CVS treatment on adrenal weights, evening OT mRNA levels, c-Fos-*ir* expressing cell numbers and affective behaviors were examined using Student's t-tests. For evening CRH mRNA levels, effects of CVS were assessed with Welch's t-test. Plasma CORT levels and morning mRNA expression levels were analyzed by two-way (CVS treatment X restraint stress) ANOVAs. All post hoc analyses were performed with the Fisher's Least Significant Difference test. Significance was set at $p<0.05$.

4.4 Results

Experiment 1. Effect of CVS on the morning HPA axis response to a novel, acute stressor

To initially assess the influence of CVS on the activity of the HPA axis, we compared weights of adrenal glands collected from CVS-treated versus control subjects. Adrenal weight was significantly increased following CVS when expressed as percentage of body weight [$p<0.001$] (Figure 4.1).

We further examined morning plasma CORT levels immediately following a novel, 20-minute restraint stressor versus basal, non-stress levels in CVS- and control-treated subjects. Regardless of previous exposure to CVS, restraint stress significantly elevated plasma CORT levels. Accordingly, a two-way (CVS x restraint stress) ANOVA showed a significant effect of restraint stress [$F(1,32)=641.5$; $p<0.0001$], but not a CVS or interaction effect (Figure 4.2).

Morning neuropeptide expression in the PVN was significantly altered by restraint stress, but in a neuropeptide-specific manner. Two-way ANOVAs revealed that there was no acute stressor effect on CRH (Figure 4.3a) mRNA levels, whereas there was an effect of restraint stress to decrease AVP [$F(1,32)=4.754$; $p<0.05$; Figure 4.3b] and OT [$F(1,30)=11.14$; $p<0.01$; Figure 4.3c] mRNA levels in both CVS-treated and control females. Because we originally hypothesized that CVS would alter the HPA axis response to an acute, novel stressor, we performed post hoc analyses to examine the effect of restraint stress within CVS and control groups. Acute restraint stress significantly decreased OT mRNA in CVS ($p<0.01$) but not control females ($p=0.2139$). In contrast, restraint stress decreased AVP mRNA in control ($p<0.05$) but not CVS females ($p=0.3602$).

To determine if the effects of acute restraint stress on PVN *Ot* gene expression in CVS- and control-treated subjects aligned with changes in circulating OT protein, plasma OT levels

were also examined. Two-way ANOVA revealed a trend for restraint to decrease plasma OT in both CVS- and control-treated animals [$F(1,31)=3.987$; $p=0.0547$] (Figure 4.3d).

Experiment 2. Effect of CVS on the evening HPA axis response to an acute stressor

To examine evening HPA axis activity in CVS-treated versus control subjects, we first assessed plasma CORT levels at 1700 h after 20 minutes of restraint stress compared to basal values. A two-way ANOVA showed a significant CVS by restraint stress interaction [$F(1,25)=11.72$; $p<0.01$] (Figure 4.4). Post hoc contrasts revealed that CVS-treated females had elevated nonstress CORT levels ($p<0.05$) and decreased stress-induced CORT levels ($p<0.05$) compared to those of control females. Moreover, whereas there was a trend for restraint stress to increase CORT in control females ($p=0.0833$), CVS females exhibited a significant drop in plasma CORT in response to acute restraint ($p<0.01$).

We next sought to investigate changes in the PVN that may contribute to the CVS-dependent effects of acute stress on evening plasma CORT levels. In the evening, CVS significantly reduced PVN neuronal activation, as measured by c-Fos-*ir,* 90 minutes after onset of the 20 minute restraint stressor [$p<0.0001$] (Figure 4.5).

Experiment 3. Effects of CVS on anxiety- and depressive-like behavior and on PVN neuropeptide gene expression

Behaviors in the light/dark box and the tail suspension test were analyzed to assess the effect of CVS exposure on anxiety- and depressive-like behavior, respectively. For the light/dark box, no differences in time spent in the light compartment of the box (Control: $M=116.37\pm18.67$; CVS: $M=96.17\pm10.17$), entries into the light compartment (Control: $M=6.67\pm1.05$; CVS: $M=7.67\pm0.60$), or initial latency to enter the light compartment (Control: $M=47.37\pm4.96$; CVS:

M=50.94±10.99) were observed. In addition, no differences in the percent duration of immobility (Control: M=47.37±4.96; CVS: M=56.59±3.91) were seen in the tail suspension test.

Although no effects of CVS were observed on behaviors in the light/dark box or the tail suspension test, CVS altered neuropeptide gene expression within the PVN. Two hours after behavioral testing, there was a trend for CVS to increase CRH mRNA [Control: M=5.48±1.40; CVS: M=35.63±13.48; p=0.0969], and OT mRNA was significantly elevated by CVS [p<0.05; Figure 4.6].

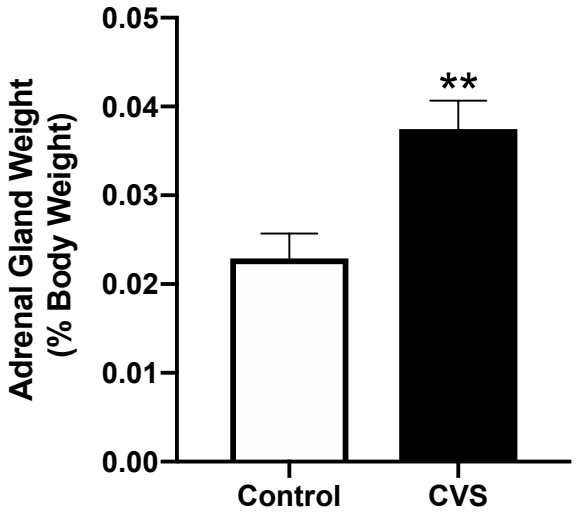

Figure 4.1. Adrenal weights (% of body weight) of intact female mice exposed to six weeks of chronic variable stress (CVS) and control subjects. Each bar represents the mean ± SEM of n=14 /group. **=p<0.001.

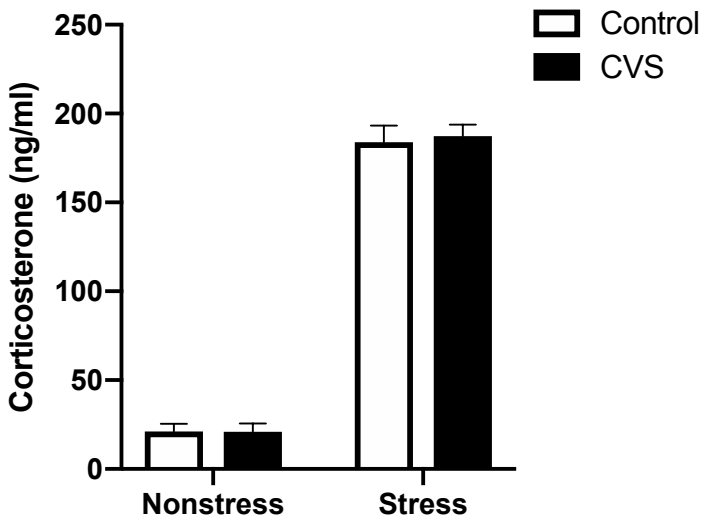

Figure 4.2. Morning (0900-1100 h) plasma corticosterone levels in control or chronic variable stress (CVS)-treated female mice collected at baseline (nonstress) or 20 minutes after initiation of restraint stress. Each bar represents the mean ± SEM of n=9/group.

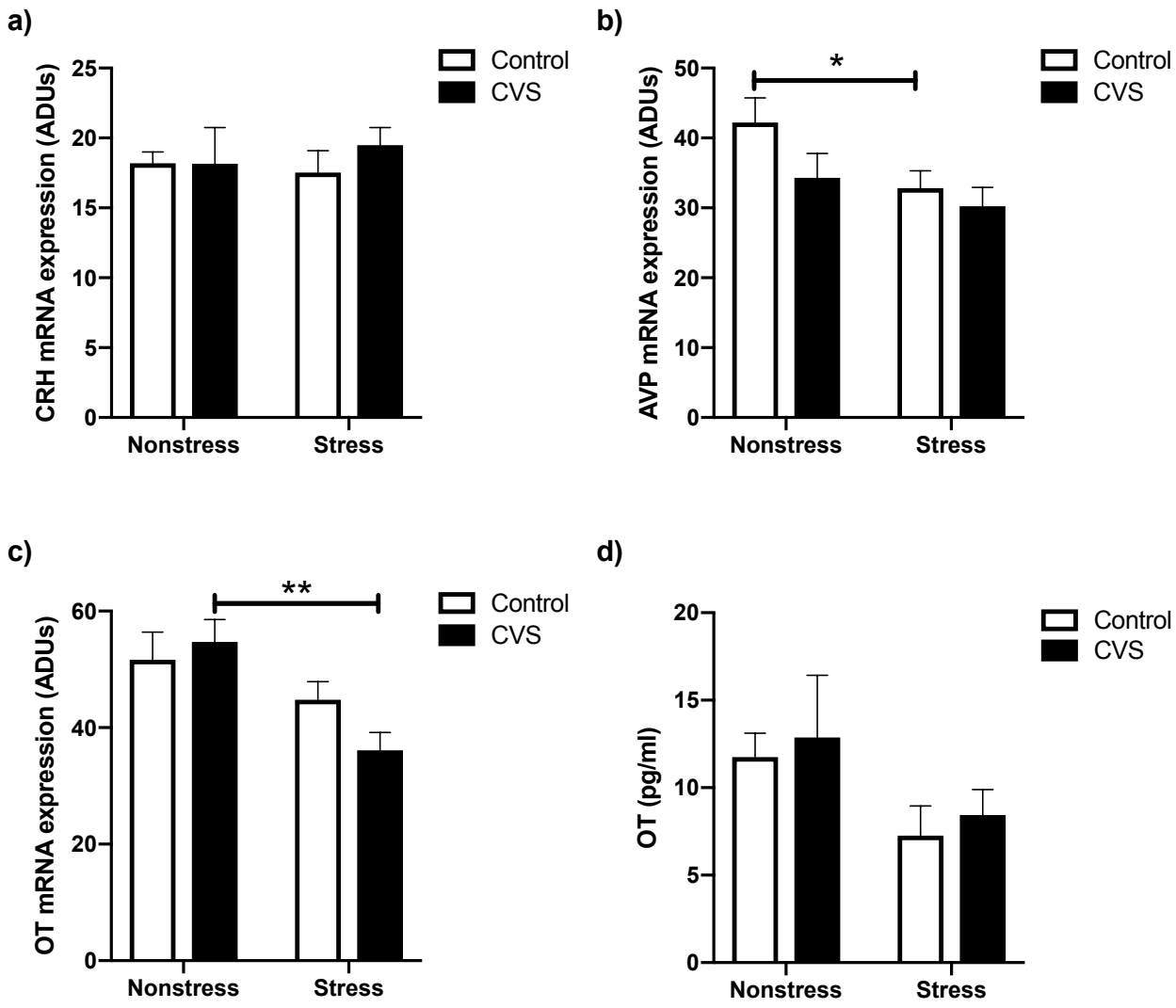

Figure 4.3. Morning (0900-1100 h) baseline (nonstress) and 20 minute restraint stress-induced levels of corticotropin releasing hormone (CRH) mRNA (a), arginine vassopressin (AVP) mRNA (b), and oxytocin (OT) mRNA (c) within the paraventricular nucleus (PVN) of female mice exposed to six weeks of chronic variable stress (CVS) versus control subjects. Panel d shows morning basal and restaint induced plasma OT levels in control and CVS females. Each bar represents the mean ± SEM of n=6-9 /group. *=$p<0.05$, and **=$p<0.01$ versus the nonstressed group of the same treatment. ADUs= Arbitrary density units.

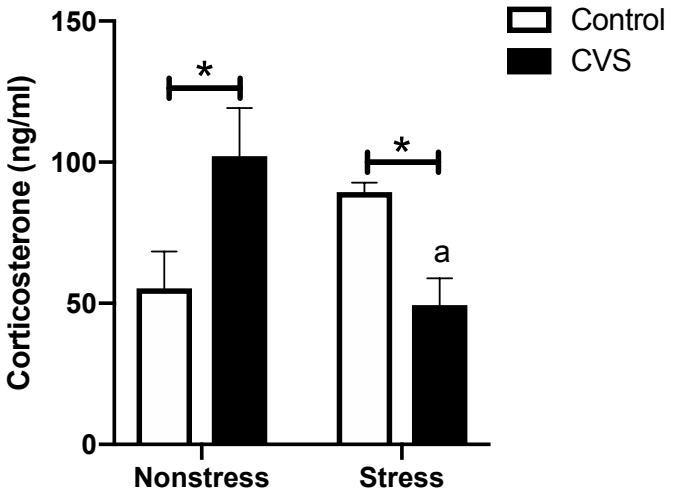

Figure 4.4. Evening (1700 h) plasma corticosterone levels after 20 minutes of restraint stress compared to nonstress values in chronic variable stress (CVS) and control females.
Each bar represents the mean ± SEM of n=6-8 p/group. *=$p<0.05$ for control versus CVS females within each treatment group. a=$p<0.01$ for CVS nonstress verus CVS stress females.

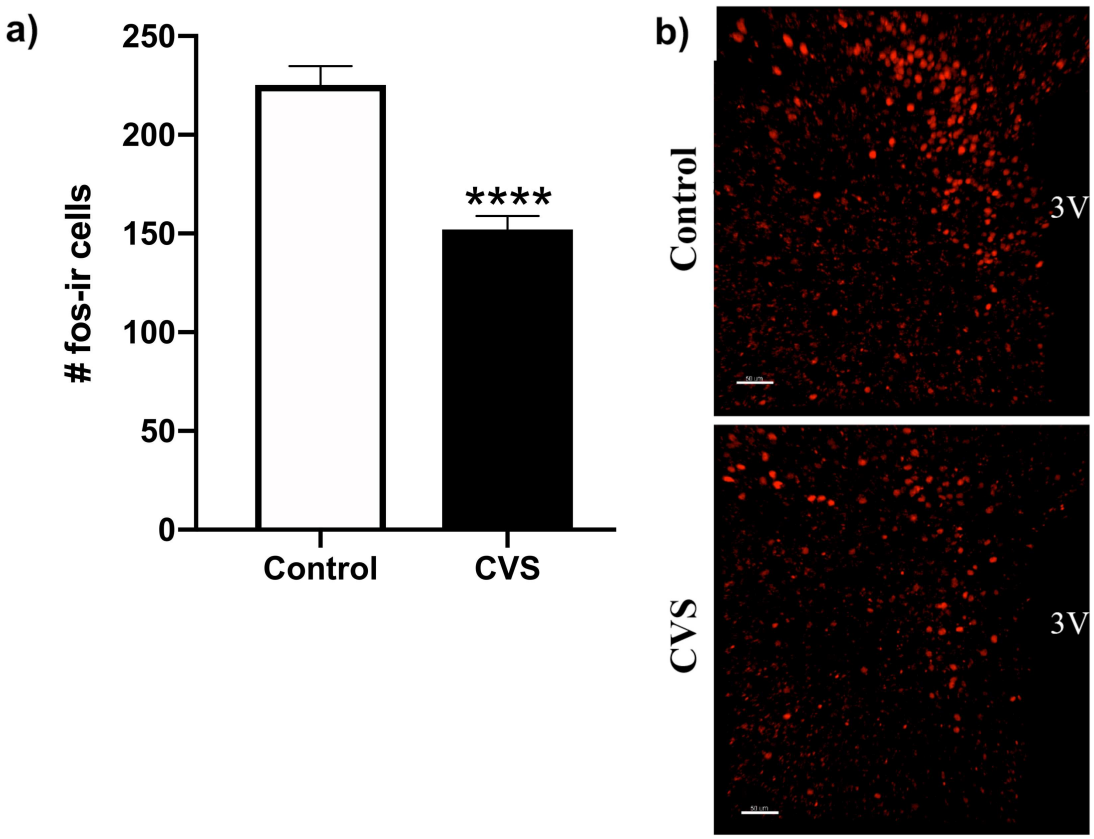

Figure 4.5. Evening c-Fos immunoreactivity (fos-*ir*) after acute restraint stress starting at 1700 h is reduced in the paraventricular nucleus (PVN) of female chronic variable stress (CVS)-treated subjects. a) Cells counts represent a single hemisphere of the PVN, averaged across 3-6 images encompassing the anterior and mid subregions. Each bar represents the mean ± SEM of 8 subjects per group. b) Photomicrographs show representative examples of fos-*ir* neurons in the PVN. 3V= 3rd ventricle. Calibration bar = 50 nm. ****= $p<0.0001$.

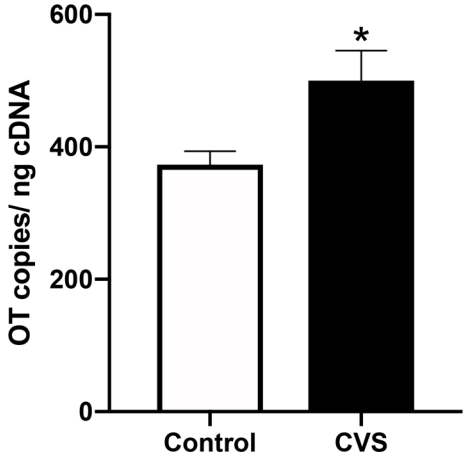

Figure 4.6. Evening (1600-1800 h) levels of oxytocin (OT) mRNA within the paraventricular nucleus (PVN) of female mice exposed to six weeks of chronic variable stress (CVS) and control subjects who all underwent testing for anxiety- and depressive-like behavior. Each bar represents the mean ± SEM of n=6 /group. *=$p<0.05$.

4.5 Discussion

CVS is an established rodent model for studying affective disorders, inducing behavioral and physiological changes in the male rodent that parallel depressive and anxiety disorders in humans (Hu *et al*, 2017; Mineur *et al*, 2006; Willner, 2017). As observed in some patients with mood disorders, CVS induces alterations in HPA axis activity indicative of hyperactivation or dysregulation (Duncko *et al*, 2001; Flak *et al*, 2011; Franco *et al*, 2016; Liu *et al*, 2017; Swaab *et al*, 2005). Despite the considerable literature on CVS effects in the male rodent, very few studies to date have investigated the consequences of CVS in females, and fewer still have examined effects in the female mouse. In the present studies we sought to characterize the effects of CVS exposure on HPA axis function in female C57BL/6 mice. The results of these studies indicate that CVS effectively induces physiological but not behavioral alterations in the female C57BL/6 mouse. CVS-treated female mice ultimately appear to show time-dependent dysregulation of the HPA axis. In the morning, females exhibited similar levels of basal CORT, which were significantly elevated by a novel, acute stressor, irrespective of CVS exposure and its effect to increase adrenal weight. Whereas acute restraint stress revealed effects of CVS on *Ot* and *Avp* gene expression, morning basal OT, AVP, and CRH mRNAs were unaltered by CVS. Alternatively, in the evening, CVS-treated females showed increased basal CORT and a hypoactive CORT response to stress, as well as decreased stress-induced neuronal activation in the PVN. Elevations in evening PVN CRH and OT mRNAs were also observed after CVS and behavioral testing. Thus, CVS may be a potent model for dysregulation of the HPA axis in the female mouse.

CVS and the morning activity of the HPA axis

Adrenal hypertrophy is a consequence of CVS commonly observed in male rodents (Ostrander *et al*, 2006; Ulrich-Lai *et al*, 2006). While a recent study by Dadomo et al. (2018) found no effect of a more severe CVS schedule on adrenal weight in female CD1 mice (Dadomo *et al*, 2018), our subjects demonstrated significant adrenal hypertrophy at the end of the study, as indicated by increased adrenal weight. Elevated adrenal weight following CVS has been linked to subregion-specific increases in cell number and size in the male rat (Ulrich-Lai *et al*, 2006). These structural changes could impart enhanced responsivity of the adrenal gland to adrenocorticotrophic hormone (ACTH), thereby increasing glucocorticoid release.

Despite the increase in adrenal weight observed following CVS in female mice, we did not detect an effect of CVS on morning basal or acute stress-induced glucocorticoid levels. Regardless of previous CVS exposure, female mice exhibited similar baseline levels of plasma CORT, which were significantly increased following 20 minutes of novel restraint stress. These results are well aligned with those of another study demonstrating that CVS and control females had similar elevations in plasma CORT after a novel forced swim stress (Dalla *et al*, 2005). Notably, our findings oppose those of previous studies conducted in the CVS-treated female rat (Liu *et al*, 2017; Uban *et al*, 2013), and, more recently, in the female mouse (Dadomo *et al*, 2018), showing elevations in basal plasma CORT levels. This discrepancy could be due to a number of factors, including varying durations of CVS exposure or the varying stressors incorporated. Additionally, subjects in the present study were exposed to isoflurane anesthesia immediately prior to collection of plasma. Isoflurane has been reported to affect CORT levels in male and female rats (Bekhbat *et al*, 2016), although C57BL/6 mice may not be as sensitive (Pomplun *et al*, 2004). Nonetheless, our findings suggest that CVS may not have a strong

influence on the morning activity of the HPA axis in female mice, as has previously been suggested in male C57BL/6 mice (Chan *et al*, 2017).

Contrary to the findings of numerous CVS studies in male rodents (Flak *et al*, 2011; Ostrander *et al*, 2006), PVN *Crh* expression at both basal and acute stress-generated levels was unaffected after six weeks of CVS exposure . While several groups have observed an elevation in PVN CRH levels in male rodents (Flak *et al*, 2011; Ostrander *et al*, 2006), research in female rats has found either elevated hypothalamic CRH mRNA (Liu *et al*, 2017), or no change following CVS (Duncko *et al*, 2001; Lan *et al*, 2015; Uban *et al*, 2013). Thus, our findings are supported by previous studies showing no effect of CVS on *Crh* expression in female rats. As CRH plays an essential role in the activation of the HPA axis and in stimulating the production of CORT, it is perhaps not surprising that we also did not observe differences in basal plasma CORT levels between CVS-treated and control female subjects.

Similar to *Crh* gene expression, morning basal PVN *Avp* expression was unaffected by CVS, as has been previously reported in female rats (Lan *et al*, 2015). However, a notable effect of CVS on the *Avp* response to a novel, acute stressor was observed. Restraint stress significantly decreased PVN AVP mRNA in control but not CVS-treated animals, suggesting that CVS inhibits the *Avp* response to acute stress. The effects of acute restraint stress on *Avp* expression are currently not well understood. Some studies in male rats have shown no alterations in AVP mRNA levels four (Harbuz *et al*, 1994; Hesketh *et al*, 2005) or six (Pinnock and Herbert, 2001) hours after acute restraint, while others report that a single period of restraint increases parvocellular PVN AVP heteronuclear RNA as early as 30 minutes and mRNA after 90 minutes or two hours (Herman, 1995; Ma *et al*, 1997a). In the present study, AVP mRNA was decreased within 20 minutes following the onset of restraint, a surprisingly rapid change for mRNA.

Accordingly, this decrease argues for an increase in mRNA degradation rather than decreased transcription. In support of this hypothesis, both cyclic adenosine monophosphate and glucocorticoids have been shown to alter AVP mRNA stability in parvocellular PVN neurons (Kuwahara *et al*, 2003) . CVS, therefore, could ultimately influence mRNA stability to inhibit the *Avp* response to acute restraint stress.

Whether or not the downstream consequences of CVS on AVP mRNA following a novel, acute stressor relate to the activity of the HPA axis remains to be determined. In humans and rats, AVP is co-expressed in some parvocelluar CRH neurons, where it acts synergistically with CRH to stimulate ACTH production by the anterior pituitary (Aguilera and Liu, 2012; Antoni, 1993). In contrast, in the mouse PVN, very little overlap of AVP- and CRH-*ir* has been found (Biag *et al*, 2012), yet a role for AVP as an ACTH secretagogue has still been identified (Lolait *et al*, 2007; Muller *et al*, 2000). The rapid changes in *Avp* gene expression we observed may ultimately alter downstream glucocorticoid production as a result. However, the reduction in AVP mRNA found following restraint stress likely occurred too quickly to impact the amount of AVP peptide available for release during the restraint (Watts, 2005). Thus, it is not unexpected that glucocorticoid levels substantially increased in both control and CVS subjects despite the effect of CVS to inhibit the *Avp* response to acute restraint stress. Rather, such CVS- and restraint stress-dependent changes in AVP mRNA may alter the accumulation of peptide that drives future glucocorticoid responses. A more thorough time course study would be necessary to demonstrate such an effect. Because we examined mRNA expression in parvocellular and magnocellular neurons collectively in these studies, we also cannot exclude the possibility that CVS alters the *Avp* response to restraint stress, with consequences for behavioral and

physiological processes outside of the HPA axis (Albers, 2012; Ludwig, 1995; McCann *et al*, 2003).

Of interest, although the restraint-induced effect on *Avp* was found in control animals only, a restraint-induced decrease in OT mRNA was found in the CVS groups only. This suggests the possibility of a switch in CVS-treated animals to a more OT-dependent mechanism of HPA axis regulation. Like *Avp*, morning basal *Ot* expression in the PVN was unaltered by CVS, paralleling our previous findings in female mice that showed no changes in PVN OT mRNA after CVS and behavioral testing (Borrow *et al*, 2018). Although restraint stress has been shown to induce activation of PVN OT neurons (Miyata *et al*, 1995), OT mRNA (Hesketh *et al*, 2005), and OT secretion (Lang *et al*, 1983) in male rats, we also observed no effect of restraint stress on OT mRNA in control animals in our studies. In contrast, CVS-treated female mice showed a rapid reduction in OT mRNA following restraint. This rapid change in mRNA, as discussed for AVP, is likely explained by increased mRNA degradation rather than reduced *Ot* transcription, but further investigation is necessary.

The effects of CVS on the *Ot* response to a novel, acute stressor likely do not immediately influence glucocorticoid production by the HPA axis, as we observed no CVS-dependent changes in restraint-stimulated CORT levels. Instead, changes in OT mRNA following 20 minutes of restraint stress may alter OT peptide stores that in turn influence future glucocorticoid production. PVN OT is not only believed to act locally to inhibit CRH neurons under basal conditions, but it also has been found to potentiate CRH-induced secretion of ACTH at the level of the pituitary (Gibbs *et al*, 1984; Neumann, 2002). Thus, if the stress-generated decrease in OT mRNA observed in CVS-treated animals ultimately results in a decrease of locally released OT peptide, then this may lead to increased glucocorticoid production relative to

controls in CVS-treated females. While such a finding would align well with previous reports of elevated plasma CORT following CVS (Dadomo *et al*, 2018; Liu *et al*, 2017; Uban *et al*, 2013), a further time course of plasma CORT levels following the 20-minute restraint stress is necessary to assess this possibility. An alternate possibility is that the decrease in OT mRNA eventually produces a decrease in OT in the general circulation, which may act on the pituitary gland to decrease ACTH and CORT synthesis and secretion. Supporting this possibility, we observed a trend for restraint stress to decrease plasma levels of OT irrespective of CVS treatment. Lastly, we cannot discount the possibility that CVS alters stress-induced *Ot* expression in magnocellular neurons to control behaviors or physiological functions beyond the HPA axis (Ludwig, 1995; Lukas *et al*, 2011; McCann *et al*, 2003).

CVS and the evening activity of the HPA axis

Although we did not observe an effect of CVS on morning basal CORT levels, we did find that CVS elevated evening levels. Thus, at least in the evening, CVS-treated females exhibited hyperactivity of the HPA axis, as has been previously suggested in female rodents (Dadomo *et al*, 2018; Liu *et al*, 2017; Uban *et al*, 2013). When challenged with an acute restraint stressor, however, CVS-treated females showed a blunted CORT response, potentially related to their evening rise in CORT levels. These findings notably contrast with the results of a previous study in female rats showing similar elevations in plasma CORT levels following a novel stressor in chronically stressed and control subjects when tested in the morning (Dalla *et al*, 2005). Such discrepancies likely are the result of differences in the time of sampling (i.e. morning versus evening). It is also possible that the blunted evening stress response we observed in our subjects is reflective of the familiarity of the stressor, as acute restraint stress is one of the manipulations used in our model. Simpkiss and Devine (2003) observed a blunted ACTH

response to restraint stress in CVS-treated male rats familiar with the manipulation, but a normal response in restraint-naïve CVS-treated subjects (Simpkiss and Devine, 2003). Alternatively, research conducted in the male rat by a different group has demonstrated a non-specific attenuation of HPA axis reactivity following CVS as indicated by a blunted corticosterone response to novel stressors (Hu *et al*, 2017). Future research will examine the effects of a novel stressor on the evening CORT response in CVS-treated subjects and will determine whether our observed blunted response is sex-specific in the mouse. Nonetheless, the blunted evening stress response did not occur in control animals in our studies, suggesting that it was not simply related to the familiarity of the restraint stressor in CVS animals.

Paralleling their pattern of evening, stress-induced glucocorticoid secretion, CVS-treated female mice had decreased neuronal activation in the PVN after acute restraint, as measured by c-Fos-*ir*. Although previous studies in female rodents are lacking, the nature of CVS's effects on stress-provoked activation of the PVN has shown inconsistencies in the male rat CVS literature. Increased (Kormos *et al*, 2016) and decreased (Ostrander *et al*, 2009) expression or no change in c-Fos-ir (Choi *et al*, 2008a) within this brain area have all been reported. Thus, the decrease in PVN neuronal activation we observed following CVS and restraint is not unprecedented and moves us closer to understanding the neurobiological mechanisms that drive the evening CORT response to stress in CVS-treated females.

<u>CVS and the morning versus evening activity of the HPA axis</u>

We cannot exclude the possibility that the differential effects of CVS on the morning versus evening activity of the HPA axis we observed are due to the presence versus absence of the restraint stressor in the CVS protocols employed. Thus, our findings must be taken with the caveat that subtle variations in the CVS protocols could drive time-dependent differences in the

activity of the HPA axis. A previous study comparing HPA function following acute restraint stress in CVS-treated male rats that were familiar and unfamiliar with the restraint stressor revealed that stressor familiarity greatly influences the ACTH response (Simpkiss and Devine, 2003). However, this study also reported no differences in the CORT response to restraint in restraint-naïve versus familiar CVS-treated subjects, suggesting that restraint stressor incorporation in the evening CVS protocol may not have altered the findings of our studies (Simpkiss and Devine, 2003). Further studies are ultimately necessary to determine if the differences in the morning and evening HPA activity we observed depend on whether or not the CVS paradigm incorporates restraint stress.

In our studies, CVS seemed to dysregulate HPA axis activity in a time-dependent manner, apparently via alterations in circadian control. This conclusion is supported by evidence of a circadian rhythm for CORT secretion, as well as for *Crh*, *Avp*, and *Ot* gene expression (Dallman *et al*, 1987; Kwak *et al*, 1993; Maejima *et al*, 2017; Muglia *et al*, 1997; Nicolaides *et al*, 2014; Watts *et al*, 2004). Furthermore, in BALB/c *male* mice, CVS has been shown to elevate and phase-shift serum CORT levels in the evening, indicating a time-dependent overactivation of the HPA axis (Takahashi *et al*, 2013). Specifically, peak CORT levels were observed later in the evening in CVS-treated versus control BALB/c mice. Thus, the elevated basal evening, but not morning, CORT levels following CVS we observed in the present studies may reflect a similar phase shift in serum CORT levels. Notably, Takahashi et al. (2013) did not observe elevated or altered rhythmicity of serum CORT in C57BL/6 male mice following CVS (Takahashi *et al*, 2013). This discrepancy could result from a sex difference in CVS effects on the circadian regulation of the HPA axis in C57BL/6 mice or from differences in the durations of the CVS protocol employed. The potentially altered CORT rhythmicity following CVS in our studies

could also be a result of sleep disruption/ deprivation, as we exposed mice to various stressors during the light cycle for an especially prolonged period. Sleep deprivation has been shown to produce increases in plasma ACTH and CORT levels (Hipólide *et al*, 2006), as well as a marked effect on the circadian secretion pattern of CORT (Retana-Márquez *et al*, 2003). Given the apparent influence of CVS on CORT rhythmicity in our studies, it seems likely that the circadian control of PVN neuropeptide gene expression is similarly disrupted, but this remains to be determined.

CVS and anxiety- and depressive-like behavior

We observed no effects of CVS on anxiety- or depressive-like behavior. While our findings may reflect a sex difference in vulnerability to CVS in terms of behavior, they may also be indicative of strain-specific effects on affective behavior. A study by Mineur et al. (2006) found that, while male but not female C57BL/6 mice showed increased immobility in the tail suspension test following CVS, time in the light portion of the light/dark box was decreased following CVS in both male and female BALB/cJ mice, but not in either sex for C57BL/6 animals (Mineur *et al*, 2006). Future studies utilizing both sexes are needed to thoroughly characterize the effects of CVS on behavioral outcomes, as well as on measures of HPA axis activity. While our subjects were tested during the dark phase, at least two hours after the last CVS stressor, it is also possible that behavioral testing during the light phase would have shown different results. A study by Huynh et al. (2011) found that female rats exposed to chronic restraint stress showed a decrease in a combined Z-score of two tests of depressive-like behavior (forced swim test and sucrose preference test) compared with unstressed females when tested during the light phase but not the dark phase (Huynh *et al*, 2011). However, the effect of chronic stress on depressive-like behavior in female rats was small, as no significant effect on

depressive-like behavior was found during either light phase when examining behaviors on each test individually (Huynh *et al*, 2011). Since HPA axis dysfunction is also associated with cardiac and metabolic disease, conditions linked to chronic stress exposure (Murphy and Loria, 2017), it may be more apt to investigate CVS exposure in female mice as a potential model for disorders outside of anxiety and depression.

Despite the relative "stress resiliency" of C57BL/6 mice when compared with other murine strains (Chan *et al*, 2017), our findings indicate that our CVS paradigm has neurobiological effects for female C57BL/6 mice, which are well-positioned to influence the activity of the HPA axis. The combination of CVS and behavioral testing elevated levels of OT mRNA in the female PVN. Given the observed increase in CRH mRNA (albeit at the trend level), this simultaneous elevation in OT mRNA may reflect an attempt to compensate for the increased activation of CRH neurons. OT is often viewed as an inhibitor of HPA axis activity (Neumann, 2002). Accordingly, a previous study conducted in male rats found that intracerebroventricular administration of OT attenuated elevated parvocellular PVN CRH mRNA levels induced by CVS, and that PVN OT mRNA levels were negatively correlated with CRH mRNA levels within this region (Zheng *et al*, 2010). Because using ddPCR precluded the analysis of magnocellular and parvocellular regions of the PVN individually in our studies, we cannot restrict our findings to the parvocellular PVN. Thus, our results support the possibility that CVS increases OT mRNA in magnocellular PVN neurons, as has previously been reported in male rats (Zheng *et al*, 2010). Although changes in mRNA levels do not necessarily reflect changes in neuropeptide secretion or neurotransmission, the observed increase in PVN OT mRNA may correlate with an increase in magnocellular OT neuronal activation and a subsequent increase in the rate of OT secretion in and around the PVN. Consistent with this interpretation,

local dendritic release of OT has been shown to influence nearby cells in a paracrine fashion, and central OT administration inhibits the CRH mRNA response to stress (Ludwig and Leng, 2006; Neumann *et al*, 2000b). However, an upregulation of local OT release can act independently of axonal release. Consequently, our findings may also indicate an increase in OT available to potentiate CRH-induced secretion of ACTH at the level of the pituitary and thereby increase HPA axis activity (Gibbs *et al*, 1984). Further investigation of how CVS influences the highly complex relationship between PVN *Crh* and *Ot* gene expression to alter HPA axis activity following behavioral testing is ultimately necessary.

To avoid the potential effects of changing gonadal hormone levels across the estrous cycle, all subjects in the present study were tested outside of the proestrous phase. Previous studies assessing female C57BL/6 or C57BL/6J mice showed that, when tested in proestrus, subjects showed decreased duration of immobility in the tail suspension test (Meziane *et al*, 2007) and increased pituitary CRH binding protein expression (Speert *et al*, 2002). In the rat, higher peak ACTH and CORT responses to stress have been reported during proestrus compared to the estrous and diestrous phases (Viau and Meaney, 1991). Given the influence of estrous cyclicity on HPA axis activity and affective behavior in the rodent, it is possible that effects of CVS on the female mouse may be altered by the stage of the estrous cycle during which they are tested. Such estrous cycle variations will be an important topic of future investigation, as they may ultimately explain some sex differences found following CVS.

While a more thorough battery of behavioral tests is needed to unveil novel effects of CVS exposure on affective behavior in the female mouse, our findings collectively suggest that the female C57BL/6 mouse may be better suited as a model of altered HPA axis function or of stress-associated autonomic disorders, such as metabolic and cardiovascular disease. CVS in our

studies ultimately appeared to dysregulate HPA axis activity in a time-dependent manner, likely via alterations in the circadian control of the HPA axis. Future studies of CVS and the circadian regulation of the HPA axis will ultimately improve our understanding of the CVS-induced HPA dysregulation thought to increase risk for stress-related autonomic disorders.

Chapter 5: Social isolation alters hypothalamic pituitary adrenal axis activity after chronic variable stress in male C57BL/6 mice

5.1 Summary

The chronic variable stress (CVS) paradigm is frequently used to model the changes in hypothalamic pituitary adrenal (HPA) axis function characteristic of many stress-related diseases. However, male C57BL/6 mice are typically resistant to CVS's effects, making it difficult to determine how chronic stress exposure may alter acute HPA function and regulation in these mice. As social support in rodents can profoundly influence physiological and behavioral processes, including the HPA axis, we sought to characterize the effects of CVS exposure on basal and acute stress-induced HPA axis function in pair- and single- housed adult male mice. Despite all subjects exhibiting decreased body weight gain and unaltered adrenal weight after six weeks of CVS, the corticosterone response to a novel, acute restraint stressor was enhanced by CVS exclusively in single-housed males. CVS also significantly increased arginine vasopressin (AVP) mRNA in the hypothalamic paraventricular nucleus (PVN) after an acute restraint stressor in single-housed males only. This was in contrast to a significant decrease in PVN AVP mRNA observed in control animals after restraint stress. Although CVS decreased oxytocin (OT) mRNA levels regardless of housing condition, socially isolated mice showed higher levels of plasma OT than all pair-housed groups, but only when they were not exposed to CVS and/or restraint. Collectively, our findings suggest that social isolation reveals effects of CVS on the HPA axis in male C57BL/6 mice.

5.2 Introduction

Although acute activation of the hypothalamic pituitary adrenal (HPA) axis enables essential, survival-oriented responses to stressful situations, (Tsigos and Chrousos, 2002) its

prolonged activation by chronic stressors can increase risk for numerous cardiometabolic and psychiatric disorders (De Kloet *et al*, 2005; Sapolsky *et al*, 2000). Thus, understanding HPA axis regulation under chronic stress conditions has potential to shed light on the pathology of some stress-related diseases. Neuropeptides, including corticotropin releasing hormone (CRH), arginine vasopressin (AVP) and oxytocin (OT), play a fundamental role in controlling the HPA axis, as they are directly synthesized and secreted by neuroendocrine neurons located in the hypothalamic paraventricular nucleus (PVN) in response to stressors (Gibbs *et al*, 1984; Whitnall, 1993). Their secretion into the hypothalamo-hypophyseal portal circulation allows for the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary and subsequent glucocorticoid secretion by the adrenal cortex. Glucocorticoids (e.g. cortisol in humans and corticosterone (CORT) in rats and mice), in turn, mobilize energy stores to facilitate appropriate body wide responses (Sapolsky *et al*, 2000). Chronic stressors can act at any of these levels of the HPA axis to disrupt its normal functioning (Flak *et al*, 2011; Herman *et al*, 1995; Ostrander *et al*, 2006).

Notably, social environment can also have profound influences on behavioral and physiological processes in rodents, including the HPA axis (DeVries *et al*, 2007; Kappel *et al*, 2017). Previous studies have shown altered basal and acute stress-induced CORT secretion in isolated versus socially housed rodents (Berry *et al*, 2012; Ros-Simó and Valverde, 2012). Furthermore, limbic regulation of the HPA axis may vary in male rats depending on housing condition, as a distinct pattern of neuronal activation has been found in group- versus single-housed males (Westenbroek *et al*, 2003a). Although few in number, studies have also begun to investigate the effect of social environment on HPA axis function following chronic stress,

revealing adrenal hypertrophy and unique limbic neuronal activation in group- versus single-housed male rats (Westenbroek *et al*, 2003b, 2003a).

In the current studies, we investigated whether social support influences the HPA response to chronic stress in C57BL/6 mice, a strain frequently used to study HPA axis regulation, but typically resistant to the effects of chronic variable stress (CVS) (Chan *et al*, 2017). The CVS paradigm involves exposure to a variety of mild stressors applied in an unpredictable manner over a prolonged period of time and effectively reproduces behavioral and physiological changes found in patients with stress-related diseases (Willner, 2017). Thus, it is advantageous for studying changes in HPA activity following chronic stress that may eventually increase stress-related disease risk. Using the CVS paradigm, we examined the effect of social versus isolation housing on the basal and stress-induced activity of the HPA axis in CVS- and control-treated male mice. We demonstrated that single-housed males predominantly exhibited altered HPA axis activity following CVS, indicated by a sensitized CORT response and an inverted PVN AVP mRNA response to acute stress. Additionally, socially isolated mice showed higher levels of plasma OT than all pair-housed groups, but only when unexposed to CVS and/or restraint. Our findings ultimately suggest that social isolation reveals the effects of CVS on the HPA axis in male C57BL/6 mice.

5.3 Materials and Methods

Subjects

Two-month-old male C57BL/6N mice were purchased from Charles Rivers Laboratories (Wilmington, MA) and maintained on a 12:12 light cycle (lights on at 0600 h) in the Colorado State University Laboratory Animal Research facility. Subjects were pair- or single- housed in one of two colony rooms and allowed to equilibrate to the Laboratory Animal Research facility

for at least one week prior to the start of experiments. Because of fighting between cage mates, 12 pair-housed animals were immediately separated and used in the single-housed cohort. Access to food and water was available *ad libitum*. All animal protocols were approved by the Colorado State University Institutional Animal Care and Use Committee and were performed in accordance with the guidelines of Colorado State University, the National Institutes of Health, and the Association for Assessment and Accreditation of Laboratory Animal Care International.

CVS and novel, acute restraint exposure

Adult male mice were exposed to CVS daily, over a six-week period, as previously described (Borrow *et al*, 2018). Briefly, subjects experienced an average of two semi-random stressors per day, including a morning and/or evening stressor administered at random times before 1200 h or after 1200 h, respectively. Stressors included three hours of occupying a cage with damp bedding, no bedding, or bedding soiled by same-sex unfamiliar mice, three hours of cage tilt (approximately 45°), one hour of exposure to cat odor, eight hours of white noise (85 dB) and overnight exposure to overhead light. Control subjects were housed in a separate colony room to prevent unintended stress exposure. Subjects were weighed at baseline and at six weeks after the onset of CVS or control conditions.

The morning (0900 h-1100 h) after the last CVS stressor, CVS-treated and control single- (n=4-7) or pair- (n=6-7) housed subjects were exposed to twenty minutes of restraint stress in a plexiglass tube placed inside their home cages, or they were left unperturbed. For the pair-housed animals, one cage mate was restrained while the other non-stressed cage mate was killed within one minute of first cage disturbance. All subjects were anesthetized with isoflurane, weighed, and decapitated immediately following the first cage disturbance or the termination of restraint. Upon decapitation, trunk blood was collected from all animals in chilled tubes containing 0.5 M

EDTA and aprotinin (4 mg/ml; Sigma-Aldrich, St. Louis, MO) and centrifuged at 3000 rpm for 12 min in a Beckman J6 centrifuge. Plasma was isolated and stored at –20°C until assayed for CORT and OT by radioimmunoassay (RIA). Brains and adrenal glands were also collected shortly following decapitation, fresh frozen and stored at -80°C.

Radioimmunoassays (RIAs)

Plasma CORT was measured as previously described (Weiser and Handa, 2009). Briefly, plasma was diluted (1:25) in PBS and plasma binding proteins were denatured at 65°C for one hour. Diluted plasma samples were incubated overnight at 4°C with rabbit anti-CORT antiserum (1:1200, Cat# 7120016, RRID:AB_2801269, MP Biomedicals, Sonon OH) and ^{3}H-CORT (PerkinElmer, Boston, MA) in 0.01 M PBS containing 0.1% gelatin. Dextran coated charcoal was used to separate antibody bound CORT from free CORT. Standard curves were constructed from dilutions of CORT (4-pregnen-11β, 21-diol-3, 20-dione; Steraloids, Wilton, NH; 5–500 ng/ml). The intra-assay coefficient of variation was less than 10%. Some control- and CVS- treated nonstresssed animals had undetectable CORT levels, and their CORT levels were recorded as the lower limit of detection (12.72 ng/ml).

To measure circulating OT levels, plasma was subjected to an extraction protocol that was adapted from a previous report (Moenter *et al*, 1991) using methanol. Briefly, 2.0 ml methanol was added to all samples and following vortex and centrifugation the supernatant was further subjected to cold precipitation (-20°C) for 24 hours. After three rounds of cold precipitation, the supernatant was dried under nitrogen at 45°C and resuspended in RIA buffer to the same volume as the starting amount. This product was then used in the protocol for an Oxytocin RIA kit (RK-051-01; Phoenix Pharmaceuticals, Burlingame, CA) following manufacturer's instructions. The extraction procedure resulted in the recovery of 69.1% of the

starting material as determined by spiking samples with known amounts of ^{125}I-oxytocin. The intra-assay coefficient of variation was 4.5%.

In situ hybridization

16-μm brain sections were cut into five series in the coronal plane using a CM3050 S cryostat (Leica, Wetzlar). Sections containing the supraoptic nucleus (SON) and/or PVN were cut at -20°C, thaw mounted onto positively charged slides (Superfrost Plus, VWR Scientific, West Chester, PA), and stored at -80°C. *In situ* hybridization was performed as previously described (Borrow *et al*, 2018). Briefly, tissue was thawed to room temperature, fixed, acetylated, delipidated, dehydrated in graded ethanols and air-dried. 48-bp oligonucleotide mRNA probes for *Crh* (5'CAGTTTCCTGTTGCTGTGAGCTTGCTGAGCTAACTGCTCTGCCCGGGC-3'), *Ot* (5'-AAGCAGGCAGCAAGCGAGACTGGGGCAGGCCATGGCGATGGTGCTCAG-3') and *Avp* (5'GTAGACCCGGGGCTTGGCAGAATCCACGGAC TCCCGTGTCCCAGCCAG-3') were end-labeled with [^{35}S] dATP using terminal deoxynucleotidyl transferase (Thermo Scientific, Waltham, MA) and added to hybridization solution at a concentration of 20×10^6 cpm/mL. Brain sections were incubated in this hybridization solution at 37°C overnight and then washed and dehydrated in a series of solutions with increasing levels of ethanol. To examine hybridization intensity, slides were exposed to X-ray film (Carestream Kodak Biomax MR, Carestream, Rochester, NY) for 8 days (CRH), 1 day (AVP), or 14 hours (OT) to generate autoradiograms. Analysis of film autoradiograms was conducted using ImageJ software (version 1.51r). Optical density was quantified bilaterally throughout the rostral-caudal extent of the PVN in three (for CRH), four (for AVP), or six (for OT) adjacent and anatomically matched sections using a template of fixed size (1.8 μm^2). Optical density representing AVP or OT mRNA was also

quantified bilaterally in two anatomically matched sections containing the SON using the same sized template. The density of exposed pixels in each half of the PVN or SON for all sections was expressed as arbitrary density units (ADUs). Background density in an adjacent area without labeling was subtracted from each measurement and resulting ADUs were averaged to obtain a single value per animal for statistical analysis.

Statistical analyses

All statistical analyses were performed using the Prism statistical program (GraphPad Software, La Jolla, CA). The effects of CVS treatment on body weights, adrenal weights and plasma CORT were examined using two-way ANOVAs. All other statistical comparisons were made using three-way ANOVAs. Post hoc analyses were performed with the Fisher's Least Significant Differences test. Significance was set at $p<0.05$. Additionally, all data were analyzed using the Extreme Studentized Deviate method (GraphPad) to detect significant outliers.

5.4 Results

Effects of CVS exposure on physiological measures in pair- versus single-housed mice

To initially assess the efficacy of our CVS model, we examined changes in body weight gain relative to baseline at six weeks following the start of the CVS protocol in control- and CVS-treated subjects (Figure 5.1). A two-way ANOVA (CVS X housing condition) revealed a significant main effect of CVS to decrease weight gain, regardless of housing condition ($F(1,43)=7.325$; $p<0.01$).

We also compared weights of adrenal glands collected from CVS-treated versus control pair- and single-housed subjects to begin our examination of CVS's effects on the activity of the HPA axis (Figure 5.2). No significant main effects of CVS or housing condition, or interaction

effects on adrenal weight expressed as a percentage of body weight (Figure 5.2a) or in total grams (Figure 5.2b) were found by two-way ANOVA.

Effects of housing condition and CVS on acute stress-induced plasma CORT

Plasma CORT levels immediately following a novel 20-minute restraint stressor were elevated in CVS- and control-treated subjects in a housing condition-dependent fashion (Figure 5.3). Baseline levels of CORT were often at or below the lower limit of quantification in pair or single-housed mice who were control- or CVS-treated. Thus, we restricted our examination to effects of housing condition on restraint stress-induced CORT in CVS versus control males. Two-way ANOVA showed a main effect of CVS to increase acute stress levels of CORT ($F(1,18)=4.810$; $p<0.05$), as well as a trend toward a significant housing by CVS interaction ($F(1,18)=3.219$; $p=0.0896$). Because we originally hypothesized that single versus pair housing would influence the CORT response to restraint, we performed post hoc comparisons of CVS versus control mice of the same housing condition. Single-, but not pair-, housed males exhibited significantly higher restraint-induced CORT levels following CVS exposure ($p<0.05$).

Effect of housing condition on hypothalamic neuropeptide responses to restraint stress and/or CVS

AVP

Depending on housing conditions and CVS treatment, *Avp* gene expression in the PVN was altered following acute restraint stress in male mice (Figure 5.4 a and b). A three-way ANOVA (CVS X housing X restraint) revealed a significant main effect of housing condition to decrease AVP mRNA levels in single-housed versus pair-housed mice ($F(1,37)=4.293$; $p<0.05$). Moreover, we found significant CVS by restraint ($F(1,37)=15.91$; $p<0.001$) and CVS by housing

by restraint interactions ($F(1,37)=5.035$; $p<0.05$). Although, PVN *Avp* expression was unaffected by restraint stress and CVS in pair-housed males, post hoc comparisons revealed that control single-housed males had significantly reduced PVN AVP mRNA levels following acute restraint ($p<0.05$). CVS single-housed males, in contrast, had significantly increased AVP mRNA levels after the restraint stressor ($p<0.01$).

To determine if the effects of CVS we observed on *Avp* gene expression were unique to the PVN, we also examined AVP mRNA in the SON following CVS and acute restraint in pair- versus single-housed males (Figure 5.4 c, d, and e). Three-way ANOVA showed a significant effect of single versus pair housing condition to decrease AVP mRNA levels ($F(1,38)=12.32$; $p<0.01$), as well as a significant interaction effect of CVS by housing by restraint ($F(1,38)=6.0$; $p<0.05$). While post hoc analyses revealed no significant effects of CVS or restraint stress in pair-housed males, there was a significant effect of restraint stress to decrease SON AVP mRNA in control, but not CVS, single-housed males ($p<0.01$).

OT

PVN OT mRNA was decreased by restraint and CVS, regardless of housing condition (Figure 5.5 a and b). Accordingly, a three-way ANOVA (CVS X housing X restraint) revealed a main effect of restraint stress ($F(1,39)=5.608$; $p<0.05$) and a main effect of CVS to decrease PVN OT mRNA ($F(1,39)=4.761$; $p<0.05$). In contrast, a significant effect of CVS by restraint by housing interaction was found for SON OT mRNA ($F(1,38)=6.097$; $p<0.05$; Figure 5.5 c and d). However, post hoc analyses revealed no significant differences between experimental groups.

Effects of acute restraint stress on plasma levels of OT in CVS and control subjects who were pair- versus single-housed were also examined (Figure 5.5e). A three-way ANOVA (CVS X housing X restraint) showed significant effects of CVS ($F(1,37)=7.323$; $p<0.05$), CVS by

housing interaction ($F(1,37)=5.072$; $p<0.05$), and CVS by housing by restraint interaction ($F(1,37)=4.285$; $p<0.05$). Post hoc analysis demonstrated that socially isolated mice showed higher levels of plasma OT than pair-housed males, but only when unexposed to CVS and/or restraint. Accordingly, levels of circulating OT were significantly higher in non-stressed control single-housed subjects than in single-housed mice that were CVS-treated ($p<0.001$), restrained ($p<0.01$), or treated with both CVS and restraint stress ($p<0.001$). OT levels in restraint- and/or CVS-treated single-housed males did not vary significantly from pair-housed groups.

CRH

Although we found no significant housing, restraint or interaction effects on PVN CRH mRNA by three-way ANOVA (housing x restraint x CVS), there was a strong trend toward a main effect of CVS to increase CRH mRNA ($F(1,30)=3.616$; $p=0.0669$; Figure 5.6).

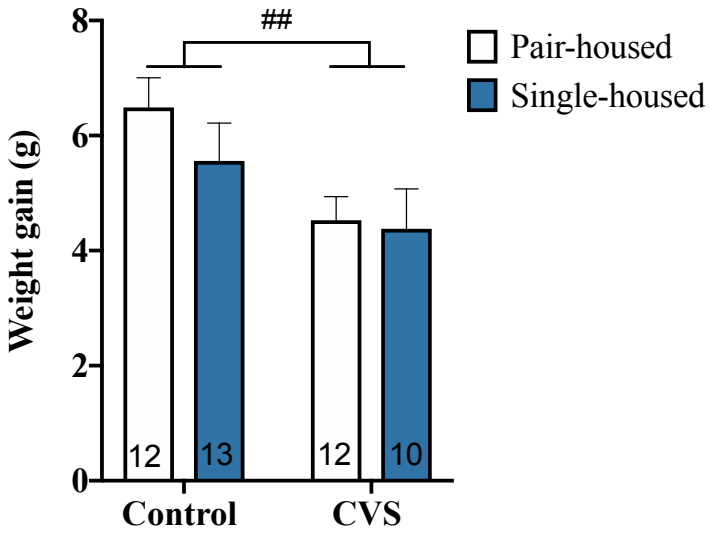

Figure 5.1. Weight gain relative to baseline weight of pair- and single-housed male mice after six-week exposure to chronic variable stress (CVS) or control conditions. Each bar represents mean ± SEM, and numbers within each bar indicate the number of animals used for each group. ##=$p<0.01$ for a main effect of CVS.

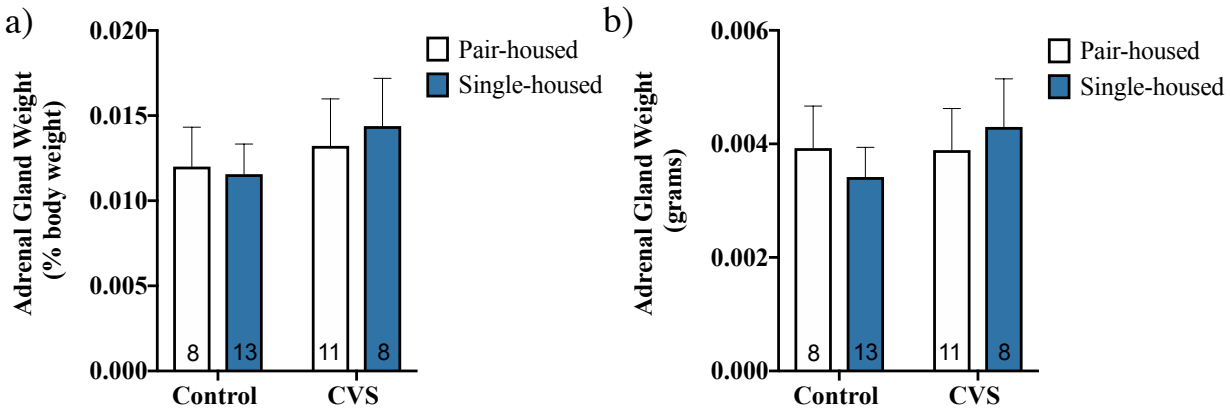

Figure 5.2. Adrenal weights (% of body weight (a); total (b)) of pair- or single-housed male mice exposed to six weeks of chronic variable stress (CVS) or control conditions. Each bar represents mean ± SEM, and numbers within each bar indicate the number of animals used for each group.

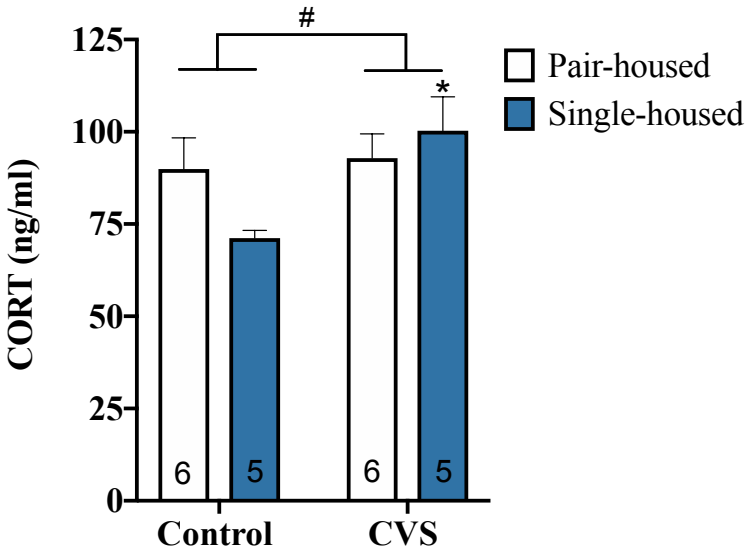

Figure 5.3. Plasma corticosterone (CORT) levels 20 minutes after the initiation of restraint stress in control or chronic variable stress (CVS)-treated pair- versus single-housed male mice. Each bar represents mean ± SEM, and numbers within each bar indicate the number of animals used for each group. Two-way ANOVA showed a significant effect of CVS (#=$p<0.05$) and a trend toward a significant housing condition by CVS interaction effect ($p=0.0896$). *=$p<0.05$ for single-housed CVS-treated versus single-housed control animals.

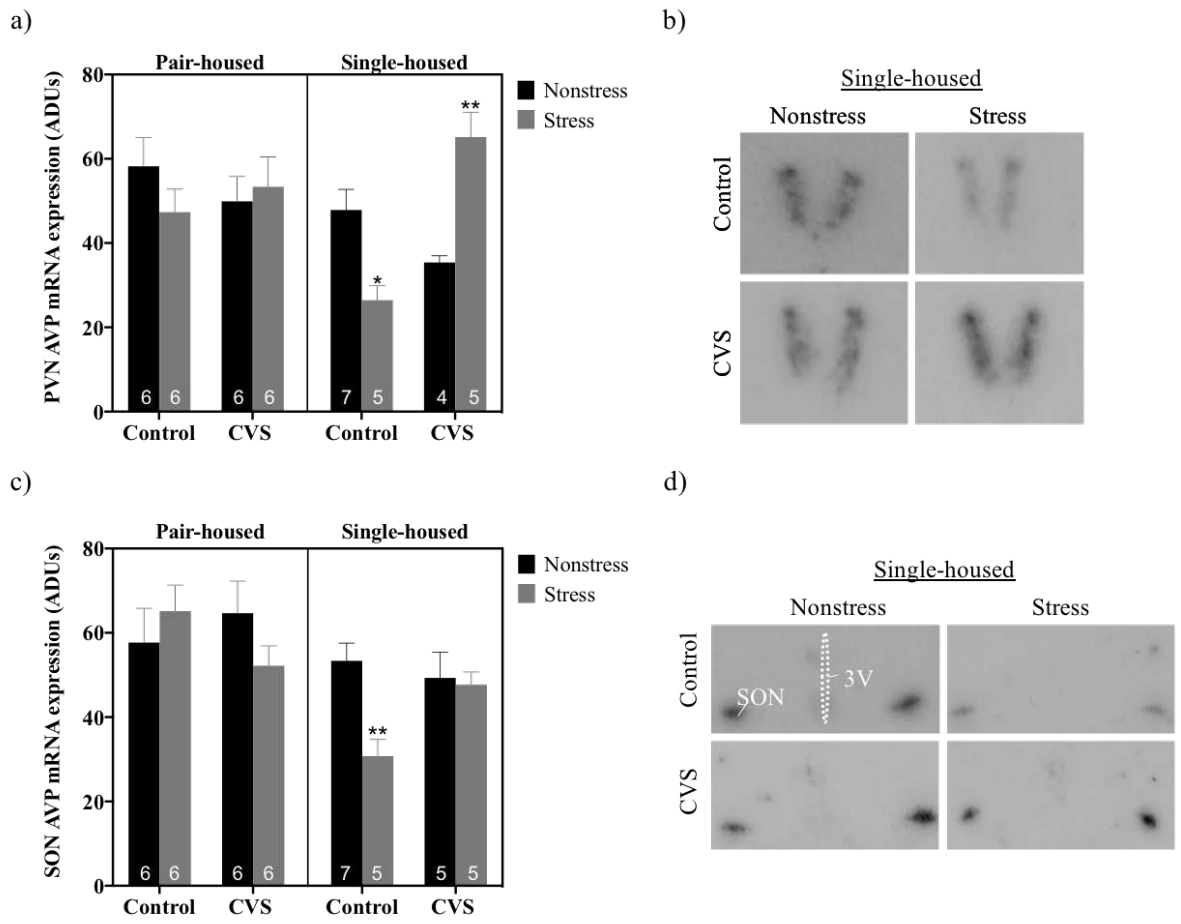

Figure 5.4. Levels of arginine vassopressin (AVP) mRNA at baseline and after 20 minutes of restraint stress within the paraventricular nucleus (PVN) (a,b) and supraoptic nucleus (SON) (c,d) of pair- versus single-housed male mice exposed to chronic variable stress (CVS) or control conditions. Arbitrary density units (ADUs) calculated from *in situ* hybridization autoradiograms of AVP mRNA are shown in panels a and c. Each bar represents mean ± SEM, and numbers within each bar indicate the number of animals used for each group. Main effects of housing condition were determined by three-way ANOVAs for PVN AVP mRNA (a; $p<0.05$) and SON AVP mRNA (c; $p<0.01$). Significant effects of CVS by housing by restraint interaction were also found for both PVN (a; $p<0.05$) and SON (c; $p<0.05$) AVP mRNA. *=$p<0.05$ and **=$p<0.01$ for stressed versus nonstressed single-housed males of the same treatement. Panels b and d show representative autoradiograms of AVP hybridization in the PVN (b) and SON (d) of single-housed males. 3V=third ventricle.

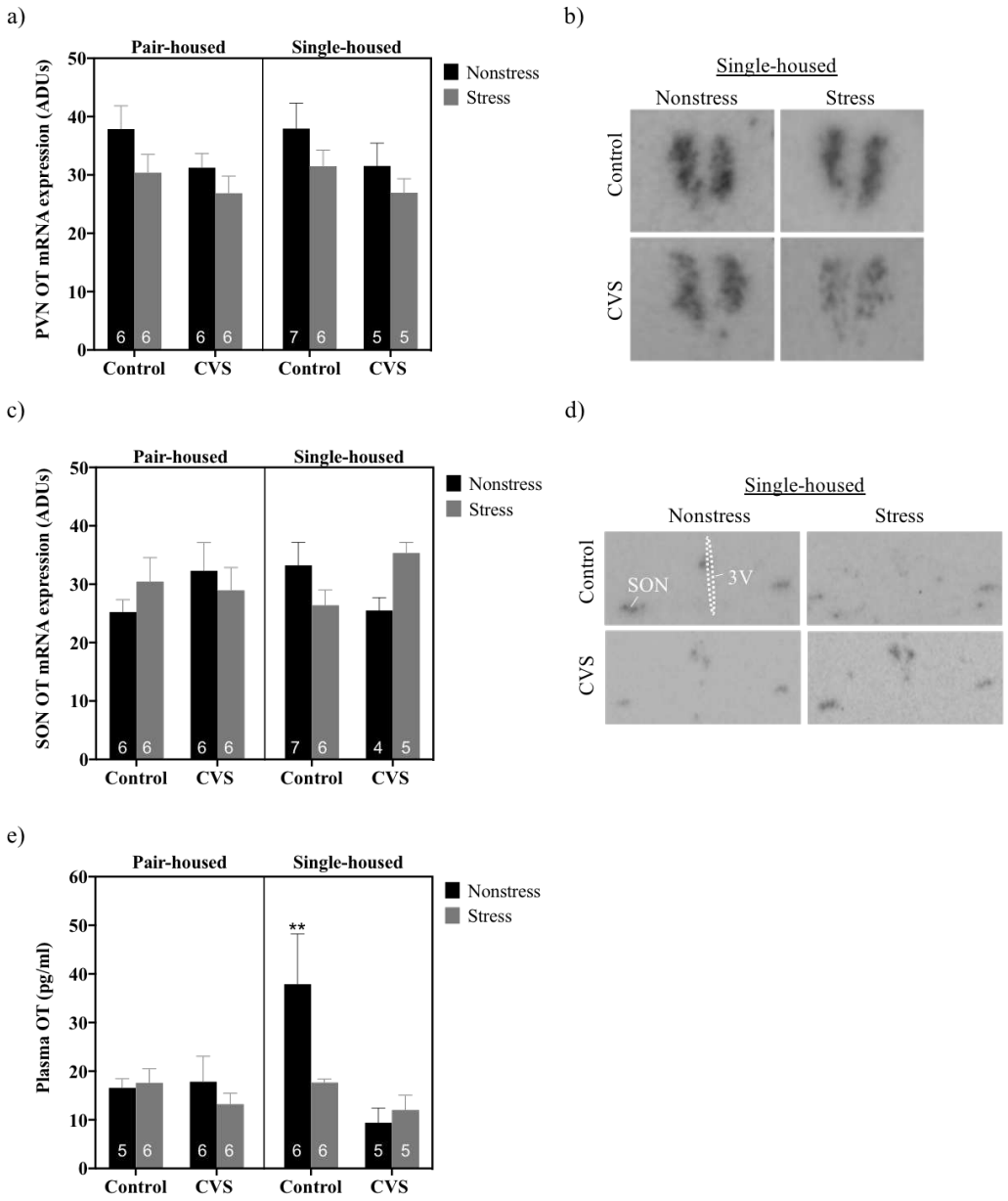

Figure 5.5. Levels of hypothalamic oxytocin (OT) mRNA and plasma OT at baseline and after 20 minutes of restraint stress in pair- versus single-housed male mice exposed to chronic variable stress (CVS) or control conditions. Panels a and c show mean ± SEM arbitrary density units (ADUs) calculated from *in situ* hybridization autoradiograms of OT mRNA within the paraventricular nucleus (PVN) (a) and supraoptic nucleus (SON) (c). Panels b and d show representative autoradiograms of OT hybridization in the PVN (b) and SON (d) of single-housed males. Panel e shows mean ± SEM plasma OT levels in pair- versus single-housed mice. Three-way ANOVAs showed main effects of restraint ($p<0.05$) and CVS ($p<0.05$) on PVN OT mRNA (a) and of CVS on plasma OT (e; $p<0.05$), as well as significant CVS by housing by restraint interaction effects for SON OT mRNA (c; $p<0.05$) and circulating OT (e; $p<0.05$) levels. In panel e, **= at least $p<0.01$ for nonstress, control single-housed male versus all other experimental groups. Numbers within each bar indicate the number of animals used for each group

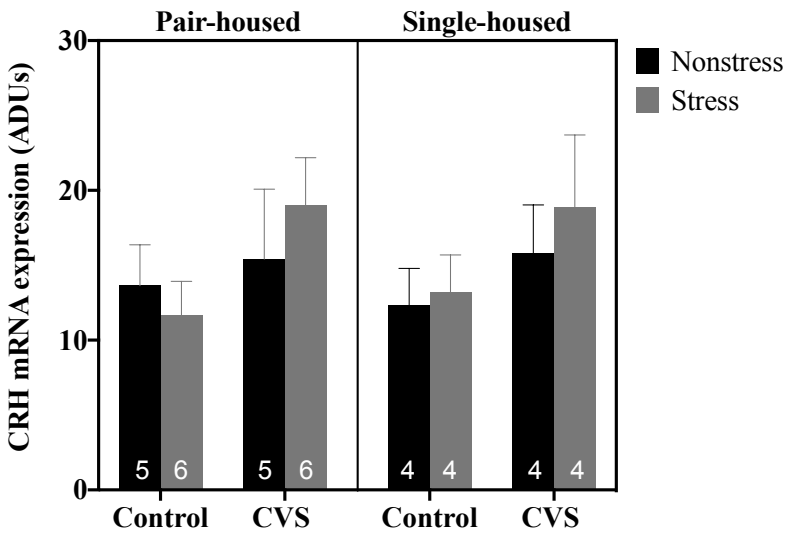

Figure 5.6. Levels of corticotropin releasing hormone (CRH) mRNA at baseline or after 20 minutes of restraint stress within the paraventricular nucleus (PVN) of pair- versus single-housed male mice exposed to chronic variable stress (CVS) or control conditions. Arbitrary density units (ADUs) calculated from *in situ* hybridization autoradiograms of CRH mRNA are shown. Each bar represents mean ± SEM, and numbers within each bar indicate the number of animals used for each group. Three-way ANOVA revealed a trend toward a main effect of CVS to increase CRH mRNA ($p=0.0669$).

5.5 Discussion

Male C57BL/6 mice are typically resistant to CVS's effects, making it difficult to determine how chronic stress may alter the acute HPA function and regulation examined in this mouse strain (Chan *et al*, 2017). As social support in male rodents can dramatically influence physiological and behavioral processes, including the HPA axis (DeVries *et al*, 2007; Kappel *et al*, 2017), we sought to determine if housing condition could influence the HPA response to CVS in C57BL/6 mice. Specifically, we characterized the effects of CVS exposure on basal and acute stress-induced HPA axis function in pair- and single-housed male mice. The results of these studies indicate that CVS alters HPA axis activity largely in single-housed, but not pair-housed, males. Although subjects exhibited decreased body weight gain, decreased PVN OT mRNA, and unaltered adrenal weight following CVS, irrespective of housing condition, the CORT response to a novel, acute stressor was enhanced by CVS exclusively in single-housed males. Likewise, we found that CVS reversed the PVN AVP mRNA response and prevented the SON AVP mRNA response to an acute stressor only in single-housed males. Furthermore, socially isolated mice showed higher levels of plasma OT than all pair-housed groups, but only when they were not exposed to CVS and/or restraint. Thus, social isolation ultimately leaves C57BL/6 male mice more sensitive to CVS-induced changes in the HPA axis.

Typically, the C57BL/6 strain is thought to be relatively stress-resistant, exhibiting lesser behavioral and physiological responses to stressors, including reduced HPA responses (Chan *et al*, 2017). Compared to BALB/cByJ mice, C57BL/6ByJ mice have reduced stress-induced ACTH and CORT levels, as well as decreased secretion of CRH and AVP at the median eminence (Anisman and Matheson, 2005; Shanks *et al*, 1990). Additionally, C57BL/6 mice, out of eight inbred mouse strains tested, were relatively resistant to behavioral effects of mild CVS,

potentially due to strain-related differences in CORT responses to stressors (Chan *et al*, 2017; Ducottet and Belzung, 2005). Alternatively, one study did report an effect of CVS to increase adrenal weight and serum CORT in group-housed male C57BL/6 mice (Monteiro *et al*, 2015). However, an eight-week protocol including a social defeat stressor rather than a four-week protocol was necessary to induce these changes (Monteiro *et al*, 2015). Thus, the near absence of CVS effects on HPA activity observed in pair-housed male C57BL/6 mice after six weeks in our studies is not unusual. As previously reported in C57BL/6 mice (Castañeda *et al*, 2011), we also observed decreased body weight in mice post CVS regardless of housing condition, supporting the efficacy of our CVS protocol (Solomon *et al*, 2010; Ulrich-Lai *et al*, 2007).

Despite their relative stress-resistance, C57BL/6 mice often exhibit altered HPA activity following social isolation. In male C57BL/6 mice, reduced plasma CORT levels and a reduced difference between the right and left adrenal glands have been reported after chronic social isolation stress, suggesting a state of isolation-provoked HPA hyporeactivity (Ieraci *et al*, 2016). Alternatively, other studies suggest a more hyperactive state of the HPA axis indicated by increased adrenal weight in socially deprived versus group-housed C57BL/6 mice (Berry *et al*, 2012). Higher CORT levels in response to a social challenge were also found in these socially deprived C57BL/6 mice (Berry *et al*, 2012). Although other studies have indicated no effect of social isolation on plasma CORT levels after four (Berry *et al*, 2012) or eight weeks (Sun *et al*, 2014) of single housing, social isolation remains well positioned to influence the HPA response to CVS in C57BL/6 mice.

Studies previously examining the effect of social environment on HPA activity under chronic stress conditions are limited, especially in C57BL/6 mice. However, one group demonstrated adrenal hypertrophy in group-housed, but not isolated male rats, as well as

housing-dependent limbic c-Fos expression (Westenbroek *et al*, 2003a, 2003b). Westenbroek et al. 2003 notably suggest that group housing enhances the effects of chronic stress in rats, whereas our findings suggest that isolation may have a greater effect, given that CVS altered HPA activity exclusively in single-housed mice. Although such discrepancies could reflect a species-related difference, an important caveat to also consider here and throughout this discussion is that pairing rodents may not be the same as group housing rodents, which may lead to increased competition for resources and a subsequent changes in the HPA axis (Beery and Kaufer, 2015; Brown and Grunberg, 1995). Nonetheless, our findings suggest that housing condition greatly influences the HPA axis response to chronic stress in C57BL/6 mice.

In rats, chronic stress exposure is often associated with altered HPA activity, indicated by adrenal hypertrophy (Westenbroek *et al*, 2003a, 2003b), altered PVN neuropeptide gene expression (Choi *et al*, 2008b; Flak *et al*, 2011; Herman *et al*, 1995; Ostrander *et al*, 2006), and elevated basal and acute stress-induced plasma CORT levels (Herman *et al*, 1995; Ostrander *et al*, 2006; Ulrich-Lai *et al*, 2007), some studies have shown an absence of such CVS-invoked changes (Bielajew *et al*, 2002; Duncko *et al*, 2001; Flak *et al*, 2011; Kioukia-Fougia *et al*, 2002; Paskitti *et al*, 2000). In the present studies, we similarly showed an absence of CVS effects on adrenal weight in both pair- and single-housed male mice, potentially reflecting the resistance of the C57BL/6 strain to CVS or the limited severity of our CVS protocol. However, we did demonstrate sensitization of the CORT response to acute stress following CVS in single housed-males. Although the two housing groups could have different temporal dynamics for observing sensitization after CVS, these findings support the possibility that, at least when socially isolated, male C57BL/6 mice exhibit altered HPA activity as is often reported in CVS-treated rats.

We also observed a striking effect of CVS to oppose the *Avp* response to a novel, acute stressor exclusively in single-housed mice. Whereas 20 minutes of restraint stress significantly decreased PVN and SON AVP mRNA in control subjects, it significantly increased PVN AVP mRNA, and had no on SON AVP mRNA, after CVS. As previous studies have reported restraint-induced alterations in AVP mRNA hours following stressor onset, our results indicate surprisingly rapid changes for mRNA (Herman, 1995; Ma *et al*, 1997b). Our findings are not unprecedented, given that rapid increases in AVP mRNA levels following restraint have been reported in 12-day-old rat pups following maternal deprivation (Dent *et al*, 2000). However, further investigation is certainly necessary to determine mechanisms for the changes in PVN AVP mRNA we observed. To influence the pool of mRNA present at any given time, restraint stress and CVS can either influence mRNA synthesis or degradation. It is possible, therefore, that 20-minute restraint enhanced mRNA degradation to rapidly decrease *Avp* mRNA levels in control subjects and that CVS reprogrammed the degradation and/or transcriptional machinery to eliminate or reverse the *Avp* response to restraint.

If or how the changes in AVP mRNA we observed in single-housed males influence the activity of the HPA axis remains to be determined. Although magnocellular AVP neurons in both the PVN and SON have been implicated in HPA axis control, the PVN, unlike the SON, also contains parvocellular AVP neurons, which directly regulate HPA activity, synergizing with CRH to stimulate ACTH production by the anterior pituitary (Herman *et al*, 2008; Lolait *et al*, 2007). Thus, the fact that CVS reversed the PVN AVP mRNA response to restraint, but only prevented the SON AVP response, may highlight varying functions for magnocellular vs. parvocellular AVP neurons during CVS and acute restraint exposure. Accordingly, in the PVN, changes in AVP mRNA in parvocellular neurons may eventually alter the accumulation of AVP

peptide stores that determine future glucocorticoid responses. AVP mRNA in magnocellular PVN neurons, like in those of the SON, may exhibit a differential response to restraint following CVS due to their greater involvement in regulating physiological functions outside of the HPA axis, such as body fluid homeostasis(Armstrong, 2015). It is also possible that the changes in *Avp* expression we observed in both regions contributed to physiological and behavioral changes outside of the neuroendocrine stress response altogether (Albers, 2012; McCann *et al*, 2003).

In these studies, we further examined effects of CVS on OT expression due to OT's known involvement in regulating HPA axis activity. Peripherally, OT can act on the anterior pituitary to stimulate ACTH production and enhance HPA output (Gibbs *et al*, 1984). An important role for PVN OT neurons to locally inhibit PVN CRH neurons and thereby limit HPA activity has also been described (Neumann *et al*, 2000a). Thus, the decreased PVN OT mRNA levels we observed following CVS, regardless of housing condition, could lead to either enhanced or inhibited HPA activity depending on whether decreased levels of PVN OT mRNA lead to decreased OT secretion into the hypophyseal portal blood and/or decreased intracerebral release (Herman *et al*, 2008; Neumann *et al*, 2000a). The strong trend we also observed for CVS to increase PVN CRH mRNA levels supports the possibility that there is less OT acting centrally to inhibit PVN *Crh* expression. A negative correlation between PVN OT and CRH mRNA levels following CVS has been previously demonstrated in male rats (Zheng *et al*, 2010). Interestingly, the decrease in OT mRNA following CVS in pair- and single-housed mice was unique to the PVN, since, in the SON, our findings support the possibility that housing condition drives the OT mRNA response to CVS and/or acute restraint. Our PVN versus SON OT mRNA findings, therefore, further support the possibility that neurons within each of these regions are differentially recruited during CVS and acute restraint depending on housing condition.

We also found alterations in circulating levels of OT in single-housed subjects. Specifically, socially isolated mice showed higher levels of plasma OT than all pair-housed groups, but only when they were not exposed to CVS and/or restraint. This elevated OT may serve a stress-buffering role during isolation, potentially compensating for an increase in HPA activity brought on by the altered housing condition, as previously suggested (Grippo *et al*, 2007). When challenged with restraint and/or CVS, then, these mice may loose the buffering capacity of OT such that decreased OT levels lead to less local inhibition of CRH neurons in the PVN and ultimately enhanced CORT secretion (Neumann *et al*, 2000a). This would be contingent upon peripheral OT secretion matching central levels, but may partly explain the augmented CORT response to a novel, acute stressor we observed in CVS-treated, isolated males. Further studies assessing this possibility are necessary. We also cannot exclude the possibilities that the CVS/restraint-induced changes in plasma OT we observed decrease HPA activity via actions at the anterior pituitary or alter physiological or behavioral processes outside the HPA axis (Gibbs *et al*, 1984; Lukas *et al*, 2011; McCann *et al*, 2003) .

Unfortunately, the volume of plasma obtained from mice was insufficient to assay other hormones beyond those that we measured. Future studies will examine changes in circulating AVP, CRH, and ACTH following restraint stress and CVS under varying housing conditions to provide a more complete picture of how social environment influences the HPA axis. Even so, the results of these studies have demonstrated CVS-invoked changes predominantly in the HPA axis of single-, but not pair-, housed male mice. Thus, at least when socially isolated, C57BL/6 male mice can be used to examine HPA function and regulation in times of chronic stress using a physiological model that is thought to be etiologically relevant for human stress-related disease risk.

Chapter 6: General Discussion

Although activation of the hypothalamic pituitary adrenal (HPA) axis is essential for an organism's adaptation and survival in the face of a stressor, its continual or untimely activation has been associated with the pathology of a number of stress-related diseases, including psychiatric disorders and cardiovascular disease (De Kloet *et al*, 2005; Sapolsky *et al*, 2000). As the experience of persistent stressful life events does not necessarily lead to the presentation of these diseases (Ebner and Singewald, 2017), an important question for modern medicine has become: what factors confer vulnerability versus resistance to the pathological effects of stress? A significant, yet historically ignored, factor is biological sex.

Support for a role for biological sex as a determinant for disease vulnerability comes partly from striking sex differences in the prevalence of many stress-related diseases (Bangasser and Valentino, 2014). For example, women are around twice as likely to be diagnosed with anxiety disorder and posttraumatic stress disorder (PTSD) (Sheikh *et al*, 2002; Tolin and Foa, 2006). The incidence of depression, as well as diseases comorbid with depression and anxiety (e.g. cardiometabolic disease and irritable bowel syndrome), is also higher in women than in men (Goldstein *et al*, 2019; Kessler *et al*, 1993; Lydiard, 2001). Men, alternatively, exhibit a higher lifetime prevalence of alcohol and drug abuse than do women (Grant *et al*, 2004; Johnston *et al*, 2003). Importantly, sociocultural factors do not fully explain sex differences in disease risk highlighting an important role for biological influences (Seedat *et al*, 2009; Seeman, 1997; Weissman *et al*, 1993).

Given sex differences in the prevalence of stress-related diseases, which are often associated with HPA axis dysregulation, the presence of sex differences in this axis have been

explored (Bangasser and Valentino, 2014; De Kloet *et al*, 2005). Although sex differences in the activity of the human HPA axis are not typically very pronounced and seem to largely depend on the stress modality, sex differences in HPA activity are more evident in patient populations. Depressed women, for instance, consistently have higher cortisol levels (i.e. main circulating glucocorticoid in humans) than depressed men, especially following stressful or negative life events (Chopra *et al*, 2009; Peeters *et al*, 2003; Young, 1995; Young and Altemus, 2004).

Activational effects of gonadal hormones also seem to influence the risk for stress-related diseases in adulthood. The sex-based risk for many of these diseases, including anxiety and depression, arises after puberty (Bale and Epperson, 2015). Additionally, women experience changes in mood and stress reactivity during key periods of estrogen fluctuation. For example, risk for postpartum depression increases during pregnancy when estrogen levels rise dramatically (Schiller *et al*, 2015). Furthermore, the menstrual cycle in women can influence sex differences in HPA axis activity (Kirschbaum *et al*, 1999).

Due to the inherent difficulty of performing human studies, most studies of sex differences in the HPA axis have used pre-clinical models in an attempt to understand sex-dependent stress-related disease pathology. The studies of this dissertation were no exception, as they utilized mice to explore factors controlling HPA activity. Thus, with the caveat that the rodent data discussed here may not translate fully to humans, this chapter will discuss how the findings in this dissertation may enhance our understanding of sex-based vulnerability to stress-related disease. First, I will discuss the findings of Chapters 2 and 3, regarding sex differences in the control of paraventricular nucleus (PVN) corticotropin releasing hormone (*Crh*) expression. I will then discuss the findings of Chapters 4 and 5, regarding HPA activity in male and female mice following chronic stress.

Sex differences in the control of PVN *Crh* expression and disease in humans

In humans, elevated cortisol levels associated with depression, especially, have been attributed to impaired glucocorticoid negative feedback on the HPA axis (Arana *et al*, 1985; Carroll *et al*, 1981). Moreover, glucocorticoid receptor (GR)-mediated suppression of CRH function has been implicated in major depressive disorder, since a combined DST/CRH test identifies most depressed individuals (Heuser *et al*, 1994a; Ising *et al*, 2005). This test uses pretreatment with the GR agonist, dexamethasone, to suppress the ability of subsequent CRH administration to increase adrenocorticotropin (ACTH) and cortisol levels, which is a typical negative feedback response. In most depressed patients, dexamethasone fails to inhibit CRH-induced HPA activity (Heuser *et al*, 1994a; Ising *et al*, 2005), suggesting a failure of negative feedback regulation. The hormonal response to CRH administration following dexamethasone pretreatment is greater in both healthy and depressed women than in their male counterparts, also supporting the possibility of sex differences in glucocorticoid negative feedback on CRH function (Heuser *et al*, 1994b; Kunugi *et al*, 2006). Thus, the studies of Chapters 2 and 3 furthered our understanding of how sex-dependent HPA regulation may contribute to sex-related vulnerability to diseases like depression, as they identified activational roles for gonadal hormones to drive sex differences in glucocorticoid negative feedback on PVN *Crh* expression.

In Chapter 2, an increase in PVN *Crh* gene expression following the removal of glucocorticoid negative feedback by adrenalectomy (ADX) for two days was found in male, but not, female mice. This more rapid response of PVN *Crh* in males depended upon gonadal androgen actions, likely via androgen receptors in neurons outside of, but important for regulating, PVN CRH neurons. If translatable to humans, these findings may suggest that males have more rapid glucocorticoid negative feedback on the HPA axis than females due to a balance

of short-term, stimulatory androgen signals and inhibitory glucocorticoid signals that converge on PVN *Crh* expression. Accordingly, under basal glucocorticoid conditions, androgens may play an important role in elevating *Crh* expression in order to prime the system for glucocorticoid negative feedback. Males may, therefore, utilize androgens to dampen the rises in glucocorticoids often found in times of chronic stress that can eventually turn detrimental. This may reduce their risk for neuropsychiatric conditions like depression as a result.

In Chapter 3, sex-dependent glucocorticoid receptor (GR)-mediated negative feedback on PVN *Crh* was identified. Adrenalectomized (ADX'd) diestrous females (low circulating estradiol (E2)) exhibited a greater decrease in PVN *Crh* expression after treatment with a GR agonist for two days than did ADX'd males, suggesting that females have more robust GR-mediated negative feedback on PVN *Crh* than do males. However, with increasing levels of E2 on proestrus, females no longer exhibited GR repression of *Crh*. Thus, increased circulating levels of E2 appear to inhibit negative feedback such that *Crh* downregulation by glucocorticoids in proestrous females is likely less than that when E2 levels are low, as well as than that in males. The results of Chapter 3 also support the possibility that sex- and/or estrous stage-dependent involvement of proposed players in the GR transcriptional machinery underlies sex differences in GR-mediated negative feedback on PVN *Crh*. Thus, increasing E2 levels lead to potential reprogramming of the GR-dependent transcriptional machinery and inhibit glucocorticoid negative feedback as a result. These effects may be acutely beneficial, enabling females to mount appropriate glucocorticoid responses to stress. However, they may also ultimately render females more vulnerable to stress-related diseases in times of chronic stress, when estrogen-impaired glucocorticoid negative feedback cannot keep up with the ever-heightening activity of the HPA axis.

Collectively, these findings support the possibility that, at least in an acute stress scenario, glucocorticoid regulation of PVN *Crh* is sex-dependent due to stimulatory androgen actions in males and inhibitory estrogen actions in females (Figure 6.1). Thus, in times of chronic stress, females may exhibit greater hyperactivity of the HPA axis, which increases their vulnerability to diseases like depression.

Future directions

A potential next step to the findings of Chapters 2 and 3 would be to examine sex-dependent expression of the GR and its transcriptional co-regulators in PVN CRH neurons specifically. Similar to the studies in Chapter 3, this could initially involve modeling GR-mediated negative feedback through the use of acutely ADX'd males, ADX'd diestrous females, and ADX'd proestrous females administered a GR agonist. Changes in gene expression of the GR and other potential players in the GR transcriptional machinery could then be assessed in PVN CRH neurons that are phenotypically distinguished from each other by alternative gene expression levels using a novel Drop-seq technology. This would highlight transcriptional regulators that warrant further investigation as participants in GR-mediated negative feedback on PVN *Crh*.

Questions geared at more directly examining the sex-/ estrous stage-dependent involvement of players in GR's regulation of PVN *Crh* could next be addressed. For example, which players are recruited to the *Crh* promoter following RU28362 treatment of ADX'd mice in a sex and/or estrous stage-dependent fashion? Are these players also necessary for GR-mediated negative feedback on PVN *Crh* depending on sex/ estrous stage? More specifically, can GR-mediated negative feedback function normally following the inducible knockdown of these transcriptional players in ADX'd males versus diestrous or proestrous females?

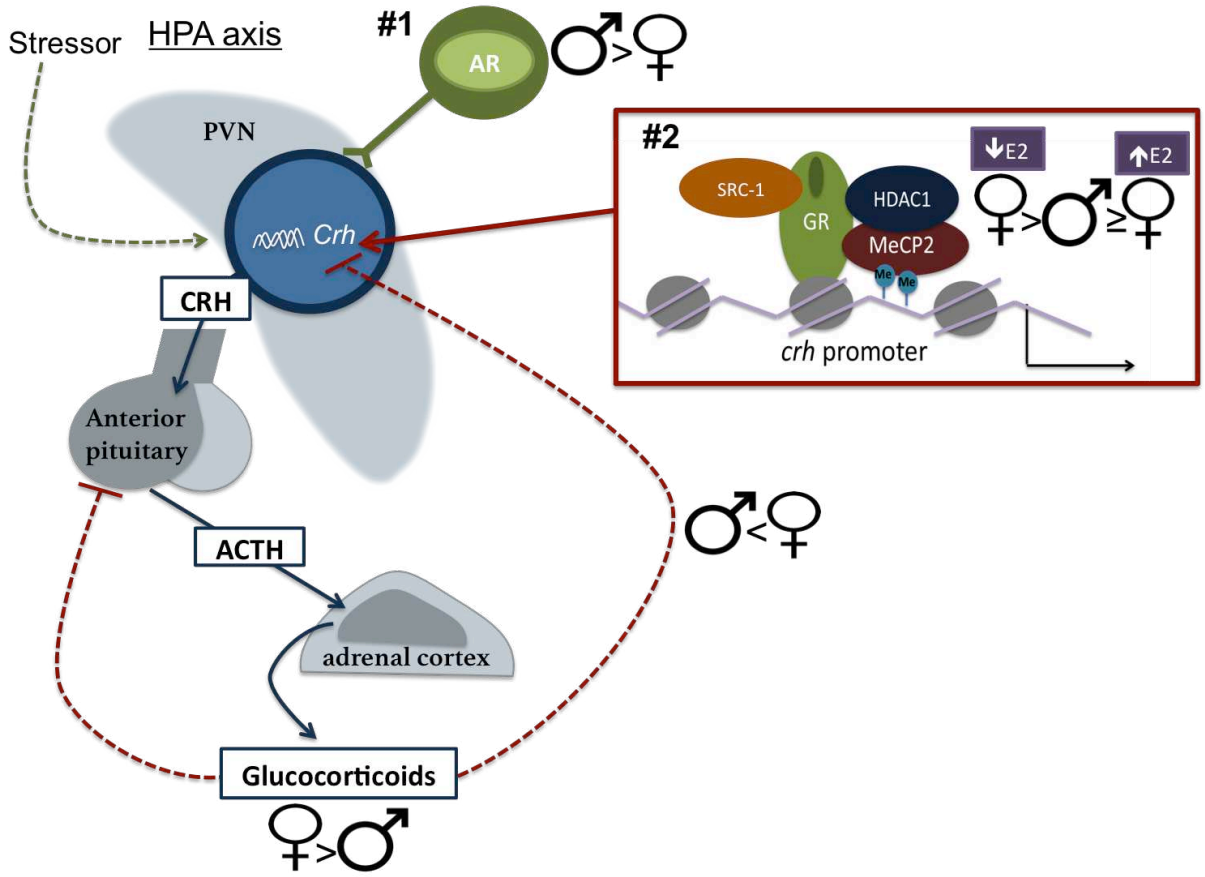

Figure 6.1. Gonadal hormones mediate sex differences in acute glucocorticoid negative feedback on PVN *Crh* to potentially contribute to sex-dependent HPA activity. The results of this dissertation support the possibility that (#1) androgens acting via androgen receptors outside of PVN CRH neurons facilitate glucocorticoid negative feedback in males such that they have more rapid glucocorticoid repression of PVN *Crh* than do females. They also support the possibility that (#2) females have lesser GR-mediated repression of PVN *Crh* than males as circulating levels of estradiol (E2) increase and alter the GR transcriptional machinery. The opposing actions of androgens in males and estrogens in females may drive sex differences in glucocorticoid negative feedback such that males are ultimately better able to limit glucocorticoid production following an acute stressor. Although such sex differences in HPA regulation may be acutely beneficial, they may contribute to sex-based HPA dysregulation following chronic stress, which may, in turn, influence sex-based vulnerability to stress-related disease. HPA axis: hypothalamic pituitary adrenal axis; PVN: paraventricular nucleus; CRH: corticotropin releasing hormone; ACTH: adrenocorticotropin; GR: glucocorticoid receptor; AR: androgen receptor; SRC-1, MeCP2 and HDAC1: potentially sex and/or estradiol dependent players in the GR transcriptional machinery; E2: estradiol.

Studies using a cell line derived from PVN CRH neurons to examine the recruitment of transcriptional regulators to the *Crh* promoter, as well as their necessity for GR-mediated negative feedback, would be beneficial to initially screen for players that warrant further exploration *in vivo*.

It would also be interesting to examine how increased levels of circulating E2 influence the involvement of players in GR's regulation of *Crh*. Are there interactions between the GR and estrogen receptors (ERs) in PVN *Crh* neurons? Although limited co-expression of ERs exists in mouse PVN CRH neurons (Oyola *et al*, 2017), the possibility remains that crosstalk occurs between GR regulatory mechanisms and ER regulatory mechanisms. If not directly in PVN CRH neurons, then where does increased circulating E2 act in female mice to influence GR's regulation of PVN *Crh*? First steps toward answering this could include the identification of ER-expressing neurons that project to CRH neurons in the PVN using recombinant adenoviruses (rAAV) in transgenic mice and immunohistochemical approaches. Eventually, specific ER-expressing neuronal populations could be activated or inhibited using chemogenetic approaches to assess their importance for modulating glucocorticoid negative feedback. Studies of this nature would be an equally important next step for the studies in Chapter 2 of this dissertation, as they would examine how androgens acting via androgen receptors facilitate the response of PVN *Crh* to ADX in male mice.

Paired with the findings of this dissertation, the results of such future investigations will ultimately bring us closer to understanding the very complex biological factors that influence sex-biased disease vulnerability.

HPA activity in male and female mice following chronic stress and disease in humans

As chronic variable stress (CVS) is a physiological model that is more etiologically relevant for human disease risk, Chapters 4 and 5, examining HPA dysregulation following CVS in mice of both sexes, may help further our understanding of sex differences in stress-related disease vulnerability. An unfortunate limitation of these studies is that no direct comparisons between males and females were made. Nevertheless, alterations in the HPA axis of females following CVS were observed that were not evident in their male counterparts. CVS-treated females, but not males, exhibited adrenal hypertrophy and a potential switch from an arginine vasopressin (AVP) to an oxytocin (OT)-dependent mechanism of HPA axis regulation following acute stress. Additionally, CVS seemed to induce a shift in the circadian rhythm of glucocorticoid secretion, such that elevated evening, but not morning, basal corticosterone (CORT) levels were present following CVS in females. This may represent another sex-dependent effect of CVS, as CVS-treated male C57BL/6 mice examined previously did not exhibit elevated or altered rhythmicity of serum CORT (Takahashi *et al*, 2013). While studies more directly examining HPA function and circadian regulation after CVS in males versus females are needed, female C57BL/6 mice appear to exhibit greater HPA dysregulation than their male counterparts following CVS. To some extent, these changes may be essential for meeting the physiological demands of chronic stress. However, at some point, these may also contribute to the increased vulnerability of females to many stress-related diseases.

C57BL/6 males appeared resistant to the effects of CVS on HPA activity as is typical for the C57BL/6 strain (Chan *et al*, 2017) . However, the studies of Chapter 5 demonstrated a striking effect of social isolation to reveal a CVS-induced alteration in HPA function. Specifically, CVS enhanced the CORT response to acute restraint in single- but not pair-housed

males. It also altered responses of PVN AVP mRNA and circulating OT to acute stress in single-housed males exclusively. Such changes collectively support a dysregulated state of HPA activity following CVS in isolated males that may be initially adaptive, but eventually increase risk for stress-related diseases. As social environment in humans can certainly influence drug and/or alcohol abuse, which are related to HPA dysregulation and are more common in men than in women, CVS-treated socially isolated males may be an ideal model for addiction-related pathology.

Although glucocorticoid negative feedback was not examined specifically in these chronic stress studies, the present results support the possibility that negative feedback is altered in times of chronic stress. The potential shift in the circadian peak glucocorticoid secretion during CVS observed in females could result partly from altered mechanisms of glucocorticoid negative feedback. Such feedback regulation on PVN *Crh* expression has been implicated in the control of both the ultradian and circadian rhythms (Gjerstad *et al*, 2018). Thus, PVN *Crh* regulation by glucocorticoids in times of chronic stress certainly warrants further investigation as a mediator of sex-based HPA dysregulation. Given that healthy and depressed women exhibit differential GR-mediated repression of PVN *Crh* compared to their male counterparts, it is critical that the sex-dependent mechanisms underlying this regulation during chronic stress be investigated (Heuser *et al*, 1994b; Kunugi *et al*, 2006).

Future directions

To expand upon the results of Chapters 4 and 5, it would be of interest to examine sex differences in glucocorticoid negative feedback on PVN *Crh* expression after chronic stress. This could initially involve measuring changes in *Crh* expression at multiple time points during/ after a novel, acute stressor in CVS-challenged versus control male and diestrous female mice. In

control animals, CRH mRNA should increase during the acute stressor and eventually decrease as a result of glucocorticoid negative feedback. E2 and/or chronic stress likely alter this CRH response to an acute stressor, but this remains to be determined. Given that CVS largely altered HPA function in isolated rather than pair-housed males in Chapter 5, it would be apt to examine both pair- and single-housed males and females in these studies.

It would also be of interest to examine differences in the necessity of the PVN GR for glucocorticoid negative feedback after a novel acute stressor in CVS-treated male and female mice on different estrous stages using an inducible knockdown approach. To begin to examine sex- and estrous stage-dependent changes in the GR transcriptional machinery, it would then be interesting to use to the Drop-seq approach to look at the molecular profile of PVN CRH neurons following CVS.

Collectively, the findings of such studies would help us to further understand how sex differences in HPA regulatory mechanisms may render one sex more vulnerable to stress-related disease following chronic stress.

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